

METHANE FERMENTATIONS: END PRODUCT INHIBITION, THERMOPHILIC METHANE FORMATION AND PRODUCTION OF METHANE FROM ALGAE

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Department of Technical Microbiology

Lund 1981

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OF METHANE FROM ALGAE

av

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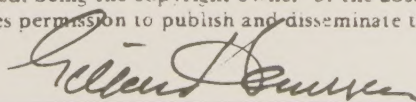
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Title and subtitle Methane Fermentations: End Product Inhibition, Thermophilic Methane Formation and Production of Methane from Algae.			
Abstract <u>End product inhibition</u> in methane fermentations on glucose and fatty acid substrates was studied in batch fermentations with enrichment cultures. Effects of pCO_2 and pCH_4 (0-1 atm) on kinetics and yields were determined. Propionate/butyrate oxidation by acetogens were the slowest steps while CH_4 -production from acetate was 3-10 times faster. CO_2 inhibited acetate and propionate degradation; rates were 4 times faster at low pCO_2 compared to 1 atm CO_2 . Butyrate degradation was not much affected. pCO_2 affected methane yields with 20-30% lower yields in 1 atm CO_2 compared to low pCO_2 . To obtain optimum rates and yields fermentations should be carried out at around 0.2 atm CO_2 . CH_4 was not significantly inhibitory. Thermophilic methane fermentation. Adaption of mesophilic ($35^\circ C$) cultures to thermophilic conditions ($55^\circ C$) proceeded rapidly suggesting that some mesophiles may switch to thermophilic conditions rather than there is a special thermophilic flora. pCO_2 caused the same degree of inhibition as in mesophilic cultures. Temperature maximum for acetate degradation was around $65^\circ C$ at high pCO_2 but considerably higher at low pCO_2 suggesting CO_2 -effects on cell membranes. In N_2 -environment methane production from glucose was possible at temperatures up to $85^\circ C$. Methane production from marine, green macro-algae was studied in semi-continuous fermentations, started with a mixed inoculum from sediments, sewage sludge, rumen contents etc. Ground algae without any additions were used as substrate. Retention times down to 12 days were suitable. Gas yields were 250-350 ml CH_4 /g VS added and the VS-reduction 50-55%. No advantages were found with a thermophilic process.			
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CONTENTS

	page
PREFACE	1
INTRODUCTION	2
METHANE FERMENTATIONS - A LITERATURE REVIEW WITH EMPHASIS ON THE BIOLOGICAL ASPECTS	4
Introduction	6
Ecology of methane fermentations	7
Microbiology and biochemistry of methanogenesis	12
Environmental factors and fermentation parameters	18
Reactor design	26
Kinetics of methane fermentations	29
SUMMARY AND DISCUSSION OF RESEARCH PAPERS	35
End product inhibition in methane fermentations (I-IV)	36
Thermophilic methane fermentation (IV)	53
Methane production from green macro-algae (V)	57
Conclusions	63
ACKNOWLEDGEMENTS	66
REFERENCES	68
RESEARCH PAPERS I-V	

PREFACE

This thesis is the result of work on methane fermentations carried out at the Department of Technical Microbiology, Chemical Centre, Lund, Sweden, during the period 1977-1981. The work has resulted in five papers which are referred to in the text by their Roman numerals:

- I Hansson G (1979). Effects of Carbon Dioxide and Methane on Methanogenesis. *European J Appl Microbiol Biotechnol*, 6:351-359
- II Hansson G, Molin N (1981). End Product Inhibition in Methane Fermentations: Effects of Carbon Dioxide on Fermentative and Acetogenic Bacteria. (*European J Appl Microbiol Biotechnol*, in press)
- III Hansson G, Molin N (1981). End Product Inhibition in Methane Fermentations: Effects of Carbon Dioxide and Methane on Methanogenic Bacteria Utilizing Acetate. (*European J Appl Microbiol Biotechnol*, in press)
- IV Hansson G (1981). Methane Production from Glucose and Fatty Acids at 55-85°C: Adaption of Cultures and Effects of pCO₂. (*European J Appl Microbiol Biotechnol*, submitted)
- V Hansson G (1981). Methane Production from Marine, Green Macro-Algae. (*Resource Recovery and Conservation*, submitted)

INTRODUCTION

In methane fermentations various organic materials are degraded to methane and carbon dioxide by complex microbial cultures during anaerobic conditions. The process is of great ecological significance and occurs in many well-known anaerobic habitats such as marshes, sediments and the rumen. Methane fermentations are known since long; methane formation in sediments was discovered in the 18th century and anaerobic digestion has been used by man since the end of the 19th century.

The interest in applications of methane fermentation has increased dramatically during the last 10 years. Applications range from treatment of dilute waste-waters to energy production from wastes and energy crops. There are several reasons for the great interest in methane fermentations: Almost all kinds of organic materials, simple and soluble compounds as well as complex polymers, may be utilized as substrates for methane production. Anaerobic digestion may be carried out in simple, inexpensive reactors during non-sterile conditions. Only small amounts of biomass is produced, which is of value in anaerobic waste-water treatment. In energy production applications the great net energy yield obtainable in this process is of vital importance.

Some important examples of present and potential applications of methane fermentations are:

- anaerobic digestion of sludge in municipal waste-water treatment has been used since long
- methane production from manure and other farm wastes is today practised in a very large number of small-scale digestors, especially in China and India. This process is also receiving great interest in Europe and the U.S.
- anaerobic treatment of industrial waste-waters is a rapidly growing full-scale application.
- municipal solid wastes as well as municipal waste-waters may be treated anaerobically in the future

- methane-production from energy crops such as sugar-beets, lucerne, grasses, leaves and algae has a very large future potential

The present and potential applications of methane fermentation require a detailed understanding of the process. Therefore considerable research and development efforts are necessary on many aspects of the process ranging from basic microbiology and biochemistry to engineering and economics. As for the biological aspects much research has been carried out and is going on in many countries. Knowledge on ecology, microbiology, biochemistry, kinetics and fermentation control of methane fermentations has increased considerably during the last decade but is still far from sufficient - mainly due to the complexity of the process. A brief literature review with emphasis on biological aspects of methane fermentations is given below.

Some of the most important factors in methane fermentations are the rate of methane production and the yield of methane. In this thesis the influence of some fermentation parameters on rates and yields have been studied. The main purpose was to study end product inhibition, i.e. inhibiting effects of the product gases CO_2 and CH_4 . End product inhibition is well-known in many fermentations and CO_2 is known to affect many bacteria but very little and often confusing literature data are available on end product inhibition in methane fermentations. In papers I -III the effects of pCO_2 and pCH_4 on fermentative, acetogenic and methanogenic bacteria in mesophilic enrichment cultures are described and the dependence of fermentation rates and methane yields on pCO_2 and CH_4 is shown. In paper IV effects of pCO_2 in thermophilic cultures are shown and also the possibility of rapid adaption of mesophilic cultures to thermophilic conditions and the further adaption of thermophilic cultures to the extreme thermophilic range. In paper V the feasibility of producing methane from marine, green macro-algae is demonstrated and basic parameters such as gas yields and retention times are described.

METHANE FERMENTATIONS - A LITERATURE REVIEW WITH EMPHASIS ON
THE BIOLOGICAL ASPECTS

	page
INTRODUCTION	6
ECOLOGY OF METHANE FERMENTATIONS	7
Methane fermentation as a three-stage process	7
Fermentative reactions	7
Hydrogen metabolism: Interspecies Hydrogen Transfer	8
Acetogenic reactions	10
Methanogenic reactions	11
Acetate production from H_2/CO_2	11
Interactions with nitrate- and sulfate-reducing bacteria	12
Methane production in animal digestive systems	12
MICROBIOLOGY AND BIOCHEMISTRY OF METHANOGENESIS	12
Taxonomy	12
Substrates for methane formation	14
Methane production from acetate	15
Biochemistry of methane formation	15
Energy metabolism	16
Morphology and composition of cell walls and lipids	17
ENVIRONMENTAL FACTORS AND FERMENTATION PARAMETERS	18
Substrates for methane fermentations	18
Biodegradability of substrates	19
Nutrient requirements	19
Inoculum	20
Temperature	21
pH, volatile fatty acids, alkalinity	22
Redox potential	23
Gas yields	23
Inhibitors	25

	page
REACTOR DESIGN	26
Batch digestors	27
Continuous fermentors without cell-recycling	27
Fermentors with cell-retention	27
KINETICS OF METHANE FERMENTATIONS	29
Methods and problems in kinetic studies	29
Kinetics of methane production from soluble substrates	30
Kinetics of methane production from complex materials	32
Kinetics of methane production with cell recycle	34

INTRODUCTION

Bacterial methane production is a process occurring in a wide range of anaerobic environments. In marshes, bogs, salt and fresh water sediments, tundra peat and even decaying heart wood of certain trees, methane formation is the last step in the decomposition of organic matter by complex microbial ecosystems. The large reserves of natural gas, which contains about 95% methane, is the result of methane production in carboniferous swamps. The gas bubbles from marshes and bogs can ignite spontaneously and cause the blue lights known as "will-o'-the wisp". Another well-known example of methanogenic habitats is the digestive tract of animals, especially ruminants. In the rumen of the cow more than 200 liters of methane are produced daily. In this case methane is formed from hydrogen and carbon dioxide while acids such as acetic and propionic acid are resorbed by the animal. Methane production from hydrogen and carbon dioxide has also been found in geothermal springs. This autotrophic methane formation in hot springs may occur at extreme thermophilic temperatures in almost boiling water.

The amount of methane produced in natural environments is surprisingly high. Ehhalt (1975) estimated that there is a net production of 500-800 million tons of methane globally per year. The actual production may be considerably greater since some of the produced methane is utilized by methane-oxidizing bacteria. The methane released to the atmosphere is balanced by its destruction, probably by reactions with hydroxyl radicals in the troposphere. Thus, there is a considerable methane cycle constituting about 1% of the atmospheric carbon cycle.

Apart from the methanogenic habitats in nature there is a range of man-made environments producing methane. For example, municipal landfills release methane for long periods of time and may be utilized as energy sources. In anaerobic digestors various kinds of organic materials may be degraded to produce methane. A very wide range of wastes, wastewaters, crop materials etc. may be used in this way to produce energy and/or solve a disposal problem and only a few examples are mentioned here. Sewage sludge digestors are in common use since many decades. In many countries energy is produced from farm waste in simple, rural digestors, e.g. in 7-8 million digestors in China. Anaerobic treatment of industrial waste waters is becoming an increasingly important process.

ECOLOGY OF METHANE FERMENTATIONS

Methane fermentation as a three-stage process

The complete degradation of organic materials to CH_4 and CO_2 is carried out by a complex mixture of interacting microorganisms, mainly bacteria. The decomposition of complex organic compounds may be described as a three-stage process according to Fig. 1 where three main groups of microorganisms are acting: fermentative, proton-reducing acetogenic and methanogenic bacteria.

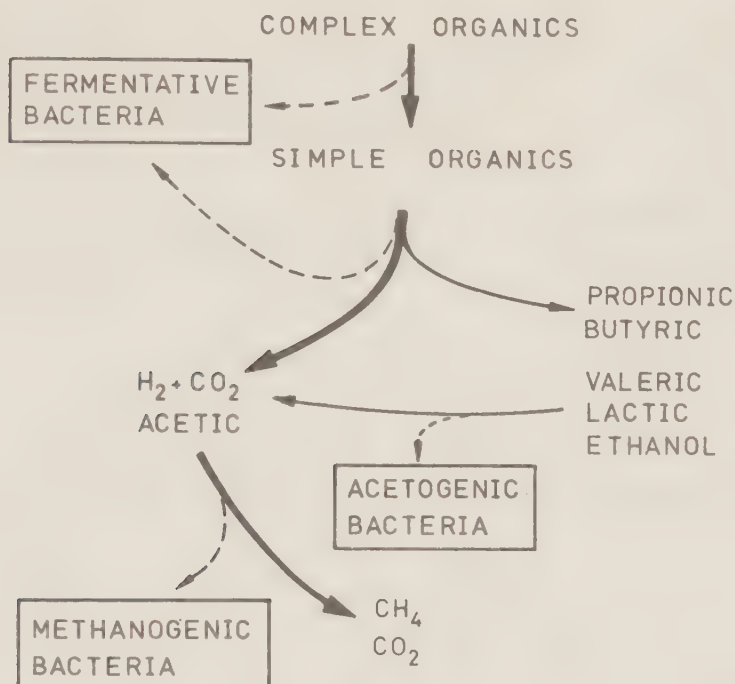


Fig 1. Main bacterial groups degrading organic compounds to CH_4 and CO_2

Fermentative reactions

The fermentation of complex organic molecules comprises two stages: the hydrolysis of compounds such as polysaccharides, proteins and lipids to corresponding monomeric units and then the further fermentation of simple compounds to short-chain fatty acids, alcohols, hydroxy acids, CO_2 , H_2 etc (Fig. 2). Also the anaerobic degradation of aromatic compounds such as benzoate and various lignin derivatives has been demonstrated (Healy and Young, 1979). Comparatively little information is available concerning the microbiology of the initial phases in anaerobic digestion. Strictly anaerobic bacteria such as Bacteroides and Clostridium predominate but also commonly found are facultative anaerobes such as streptococci and Enterobacteriaceae (Bryant 1977, Hobson et al 1974).

Hydrogen metabolism: Interspecies Hydrogen Transfer

Recent research has revealed the importance of the hydrogen partial pressure, p_{H_2} , in anaerobic fermentations (Wolin 1975). In the degradation of carbohydrates to pyruvate hydrogen is produced as NADH (Fig. 3) and NAD^+ may be regenerated in two ways, by hydrogen production or by reduction of pyruvate by NADH to various compounds. Which of these routes is followed is dependent upon the thermodynamics of the reaction

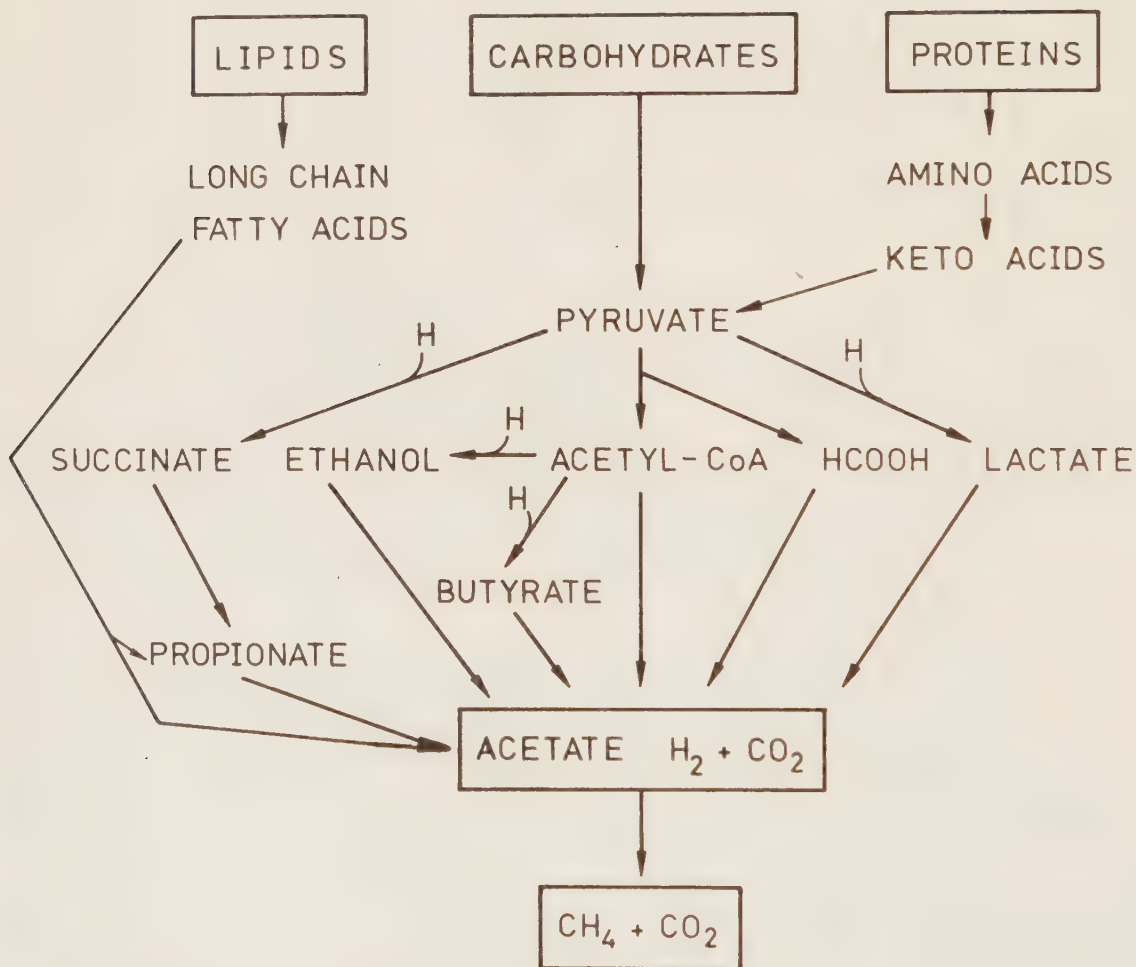


Fig 2. Schematic diagram of the decomposition of major polymers in methane fermentations

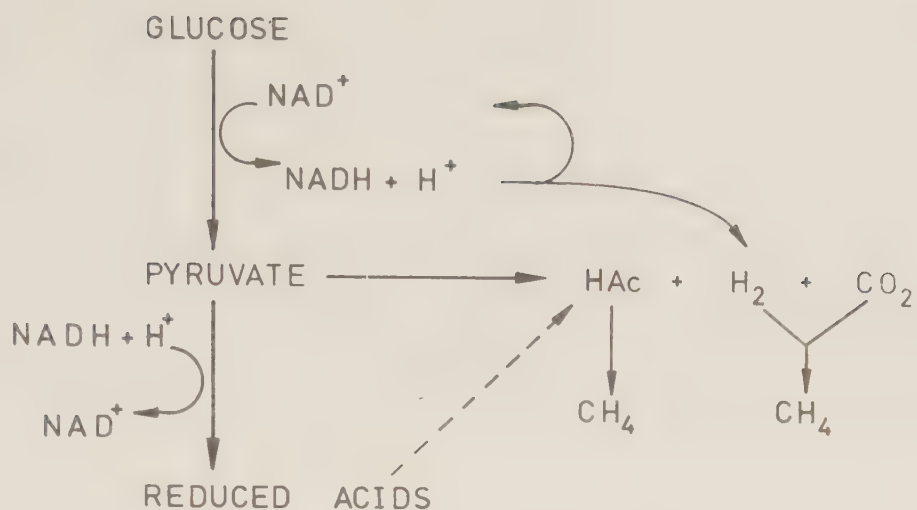


Fig 3. Regeneration of NAD^+ by production of hydrogen or reduced acids

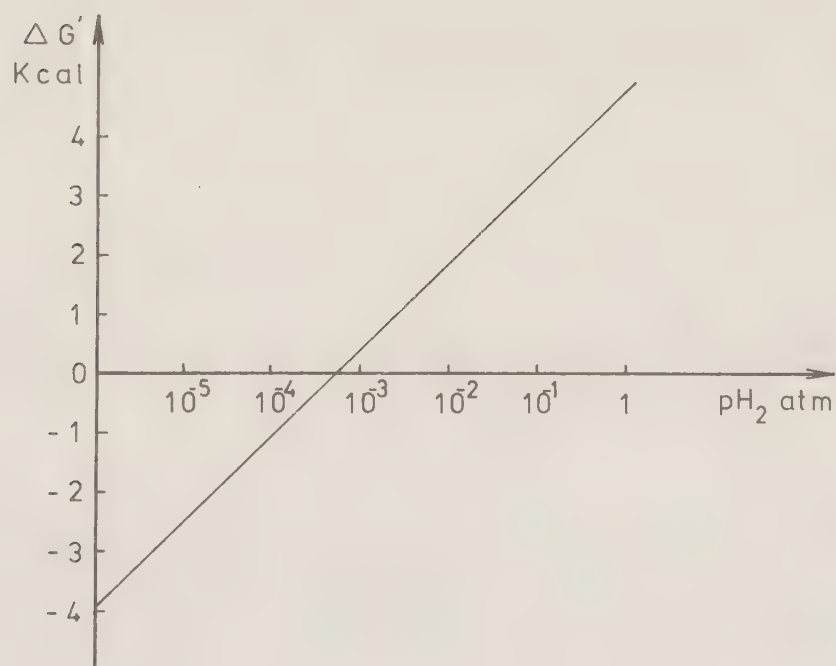


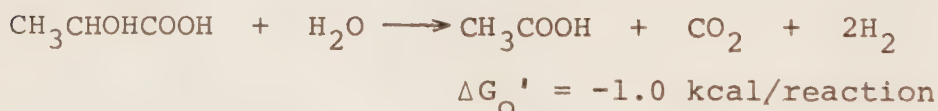
Fig 4. The influence of pH_2 on the thermodynamics of NAD^+ -formation from NADH

Under standard conditions the production of reduced acids and alcohols is favoured while the fermentation is directed towards acetate- and H_2 -production if p_{H_2} is kept below 10^{-3} - 10^{-4} atm. This is achieved by methanogenic bacteria which have the capacity to produce CH_4 from $CO_2 + H_2$. Therefore, in an efficient anaerobic digester, methane is produced with acetate and hydrogen as the main intermediates. If the methanogens are disturbed, e.g. by overloading or by toxic compounds, p_{H_2} will increase and the fermentation will be directed towards production of propionic acid, butyric acid or even ethanol and lactate. This eventually leads to a complete inhibition of the ecosystem due to low pH and inhibiting acid concentrations. Consequently, from the ecological point of view, the main function of methanogens is to remove H_2 from the digesting fluid to the atmosphere thus enabling the decomposition of organic matter in anaerobic environments to proceed.

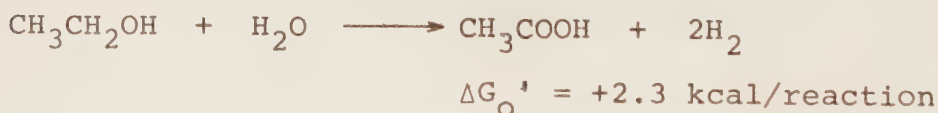
Acetogenic reactions

It was earlier believed that methane was produced from a variety of acids and alcohols, but it is now known that the only acids and alcohols utilized by methanogens are formate, acetate and methanol. Other compounds are oxidized by obligate, proton-reducing acetogenic bacteria to acetate and hydrogen which are converted to methane by methanogens. Four examples of acetogenic reactions are:

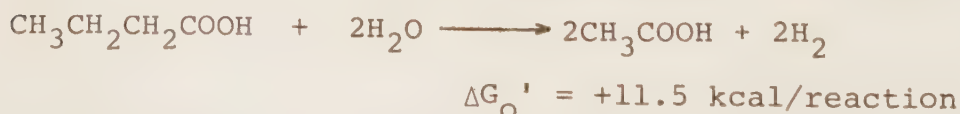
1. Oxidation of lactic acid by Desulfovibrio (Bryant et al 1977)



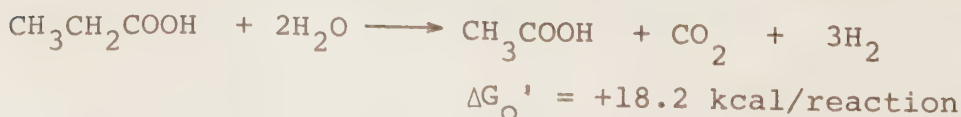
2. Oxidation of ethanol by S organism (Bryant et al 1967) or Desulfovibrio (Bryant et al 1977)



3. Oxidation of butyric acid (and higher even-numbered acids) by an organism described by McInerney et al (1979)



4. Oxidation of propionic acid by Syntrophobacter wolinii (Boone and Bryant 1980)



Of these reactions propionic and butyric acid oxidation are the most important in anaerobic digestion. For example, these acids are produced as a result of temporary disturbances of methanogens such as overloading, temperature variations and presence of toxic materials. Propionic acid is also produced in the degradation of odd-numbered fatty acids. The oxidation of propionic and butyric acid is thermodynamically very unfavourable during standard conditions and therefore it is not possible to cultivate these bacteria in pure culture. However, in coculture with methanogens or H_2 -consuming Desulfovibrio, the latter bacteria will keep an extremely low pH_2 (10^{-5} atm), necessary to "pull" the acetogenic reactions towards acetate-production. Thus, in methane production, a very efficient hydrogen consumption by methanogens is necessary for acetate production from reduced compounds to proceed.

Methanogenic reactions

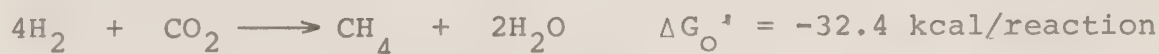
Methanogens as a group have the capacity to produce methane from $H_2 + CO_2$, CO, acetate, formate, methanol and methyl amines (Balch et al 1979). In natural environments and practical applications acetate is the most important precursor of methane, accounting for about 70 % of the methane (Smith and Mah 1966, Jeris and McCarty 1965). The rest is mainly produced from $H_2 + CO_2$ and, as outlined above, the capacity of methanogens to keep a very low pH_2 is essential to direct the activities of fermentative and acetogenic bacteria towards the production of acetate and H_2 .

The biology of methanogenic bacteria is discussed in more detail below.

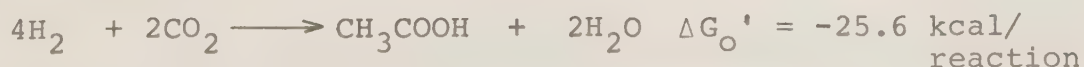
Acetate production from H_2/CO_2

Carbon dioxide is reduced by hydrogen, mainly to form methane, but acetate production may also occur (Balch et al 1979, Mah et al 1977):

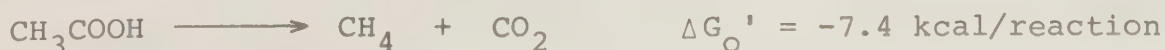
Methanogens:



Acetobacterium woodii, Clostridium aceticum and Clostridium thermoautotrophicum:



The acetate produced from $H_2 + CO_2$ would then be used by methanogens:



Bacteria producing acetate from $H_2 + CO_2$ have been isolated from muds and sewage sludge but the extent and significance of these reactions in complex ecosystems is not known. One consequence would be that one methanogenic precursor, $H_2 + CO_2$, is transferred to another methanogenic precursor, CH_3COOH , which is thermodynamically less favourable.

Interactions with nitrate- and sulfate-reducing bacteria

If nitrate and sulfate are present in substrates for methane fermentation NO_3^- , SO_4^{2-} and CO_2 will compete for electrons produced by oxidation of organic compounds. If the proper bacteria are present NO_3^- will be reduced first, then SO_4^{2-} followed by CO_2 due to the higher electron affinity of the nitrogen and sulfur atoms compared to the carbon atom (Zehnder 1978). Therefore, in complex cultures, methane production should occur only in the absence of nitrate and sulfate. For example, in sediments sulfate reduction and methane production take place at different depths. However, in most environments and applications of methane fermentations nitrate and sulfate respiration is not quantitatively important.

In the interaction between sulfate-reducers and methane-producers the former will successfully compete for H_2 and also produce toxic H_2S . On the other hand they may also act as acetogenic bacteria, producing acetate from ethanol, lactate etc. and the methanogens will benefit from this production of a methane precursor (Zeikus 1977, Mah et al 1977).

Methane production in animal digestive systems

Methane fermentations are carried out in the rumen, cecum and large intestine of many animals. The best known system is the rumen of herbivores. There is, however, a considerable difference between methane fermentations in the rumen and in other environments. The holding time of the digesting fluid is comparatively short in the rumen. Therefore slow-growing bacteria such as acetate-utilizing methanogens and propionate- or butyrate-oxidizing acetogens are diluted from the system while fast-growing methanogens growing on H_2/CO_2 are retained. Consequently, methane is produced only from H_2/CO_2 while acids accumulate. The acids are absorbed and utilized as an energy source by the animal while gases are removed by belching.

MICROBIOLOGY AND BIOCHEMISTRY OF METHANOGENESIS

Taxonomy

Recent research has led to a considerably increased understanding of methane-producing bacteria. One reason is the

improved methods of cultivating methanogens (Balch and Wolfe 1976). With the techniques now available, the cultivation of methanogens in pure culture is regarded as no more difficult than the cultivation of ordinary anaerobes.

Methanogens now appear as a unique group of bacteria, as shown in the results from recent phylogenetic approaches on bacterial taxonomy (Balch et al 1979). In this approach 16S rRNA from bacteria is analysed and compared. The ribosomal RNA is regarded as a very conserved molecule changing only slowly with time by mutations. Therefore, by comparing the composition of 16S rRNA in different organisms their phylogenetic relatedness may be established. In this way over 100 species of bacteria have been characterized, including methanogens. These studies have revealed methanogens, together with some extreme halophiles and thermoacidophiles, as unique bacteria only distantly related to other bacteria. This bacterial group, known as Archaeobacteria, is further characterized by several unique structural and biochemical properties such as different composition of RNAs, cell walls and cell membranes (Woese et al 1978). A new, detailed taxonomy of methanogens has recently been proposed by Balch et al (1979), Table 1.

Table 1. Taxonomy of methanogens

Order	Family	Genus	Species	Substrates
I	Methanobacteriales			
	Methanobacteriaceae			
		Methanobacterium		
			M. formicicum	H ₂ , HCOOH
			M. bryantii	H ₂
			M. thermoautotrophicum	H ₂
		Methanobrevibacter		
			M. ruminantium	H ₂ , HCOOH
			M. arboriphilus	H ₂
			M. smithii	H ₂ , HCOOH
II	Methanococcales			
	Methanococcaceae			
		Methanococcus		
			M. vanniellii	H ₂ , HCOOH
			M. voltae	H ₂ , HCOOH
III	Methanomicrobiales			
	Methanomicrobiaceae			
		Methanomicrobium		
			M. mobile	H ₂ , HCOOH
		Methanogenium		
			M. cariaci	H ₂ , HCOOH
			M. marisnigri	H ₂ , HCOOH
		Methanospirillum		
			M. hungatei	H ₂ , HCOOH
	Methanosarcinaceae			
		Methanosarcina		
			M. barkeri	H ₂ , CH ₃ COOH, CH ₃ OH, CH ₃ NH ₂

Substrates for methane formation

An overview of methanogenic bacteria and their substrates was shown in Table 1. Equations and thermodynamics of methane formation are summarized here (Thauer et al 1977, Daniels et al 1977, Balch et al 1979, Hippe et al 1979):

	$\Delta G_o'$ kcal/reaction
(1) $4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$	-32.4
(2) $4HCOOH \longrightarrow CH_4 + 3CO_2 + 2H_2O$	-31.2
(3) $CO + 3H_2 \longrightarrow CH_4 + H_2O$	-36.0
(4) $4CO + 2H_2O \longrightarrow 3CO_2 + CH_4$	-50.5
(5) $4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$	-75.2
(6) Various N-methyl compounds, e.g. $4CH_3NH_3Cl + 2H_2O \longrightarrow 3CH_4 + CO_2 + 4NH_4Cl$	-53.8
(7) $CH_3COOH \longrightarrow CH_4 + CO_2$	- 7.4

The reduction of CO_2 with H_2 is generally regarded as a reaction carried out by all methanogens. However, a thermophilic strain of Methanosarcina, unable to use $H_2 + CO_2$, was isolated by Zinder and Mah (1979). Furthermore, the methanogen often referred to as the "fat rod" often seen under the microscope but only recently isolated by Zehnder et al (1980) was found not to utilize $H_2 + CO_2$. When methanogens grow on $H_2 + CO_2$ the energy yield is comparatively high and consequently growth is rapid. For example, Methanobacterium thermoautotrophicum may be cultivated on $H_2 + CO_2$ in an inorganic medium with generation times of less than 3 hours (Zeikus 1977).

Except for the almost general capacity to utilize $H_2 + CO_2$ many methanogens as listed in Table 1, may also convert formate to H_2 and CO_2 and then to CH_4 (equation 2). Some methanogens may also utilize CO, either by reduction of CO to CH_4 by H_2 (equation 3) or by a disproportionating reaction (equation 4), as shown for Methanobacterium thermoautotrophicum (Daniels et al 1977). Methanosarcina barkeri is the only methanogen producing methane from methanol or various methylamines (equation 5 and 6). The utilization of formate, carbon monoxide, methanol and methylamines is not regarded to be of any significant quantitative importance in natural environments or sewage sludge digestors although anaerobic degradation of methylamines, creatin, betaine etc. may be ecologically important (Hippe et al 1979).

How CO_2 is reduced to cell constituents is not known. Key enzymes for the Calvin cycle, serine pathway, hexulose pathway, reductive pentose phosphate pathway, reductive tricarboxylic

acid pathway or acetate production from CO_2 have not been demonstrated and possibly a new pathway of autotrophic CO_2 -fixation exists in methanogens (Balch et al 1979).

As for nitrogen assimilation NH_4^+ is regarded as the major nitrogen source for all methanogens (Bryant et al 1971). Complex nitrogen sources such as amino acids, peptides, purines and pyrimidines do not appear to be required by methanogens.

Methane production from acetate

As mentioned above, acetate is the precursor of about 70 % of the methane produced in most environments, except for animal digestive systems. Thus, methane production from acetate is of great importance but still little is known about acetate-utilizing methanogens. Until recently Methanosarcina barkeri was the only methanogen known to utilize acetate as the main substrate. Many attempts to obtain acetate-utilizing methanogens in pure culture have failed or acetate decomposition has been extremely slow. The slow growth and methane production on acetate is a result of the low energy yield in acetate splitting. However, Mah et al (1977) obtained comparatively fast growth of a strain of Methanosarcina barkeri with doubling times of 24-72 hours. This organism fermented acetate at a rate comparable to those found in complex cultures in anaerobic digestors and acetate enrichments. The recently isolated "fat rod" (Zehnder et al 1980) utilizes acetate but is considerably slower with generation times around 9 days. On the other hand its substrate affinity is higher, $K_s = 0.46 \text{ mM}$ compared to $K_s = 5 \text{ mM}$ for Methanosarcina which makes it possible for the "fat rod" to compete with faster organisms in fermentations with long retention times and low acetate concentrations. Due to its capacity to form filaments the "fat rod" will be named Methanotrix soehngenii (Zehnder 1981). Another methanogen, Methanococcus mazei has been known since long, but was never obtained in pure culture. This organism is presently being characterized (Zehnder 1981) and has been found to utilize hydrogen, acetate, methanol and methylamines in resemblance to Methanosarcina barkeri.

Biochemistry of methane formation

Barker (1956) suggested a general model for the stepwise reduction of CO_2 to CH_4 by means of one or more unknown carrier molecules $\bar{X}$ (Fig. 5). In this scheme methyl groups from acetate and methanol may be donated directly to the carrier $\bar{X}$. For many years tetrahydrofolic acid and vitamin B_{12} , both known in C_1 -metabolism, were considered as possible carriers $\bar{X}$. In the 1970s however, a methyl carrier coenzyme M, 2-mercaptoethanesulfonic acid, $\text{HSCH}_2\text{CH}_2\text{SO}_3^-$, was identified by Taylor and Wolfe (1974). CoM has not been found in any other organisms than

methanogens. A cycle for CO_2 -reduction to CH_4 has been proposed (Gunsalus et al 1975, Wolfe 1979), Fig. 5, although all the steps in the cycle are not proved and other factors than CoM are involved.

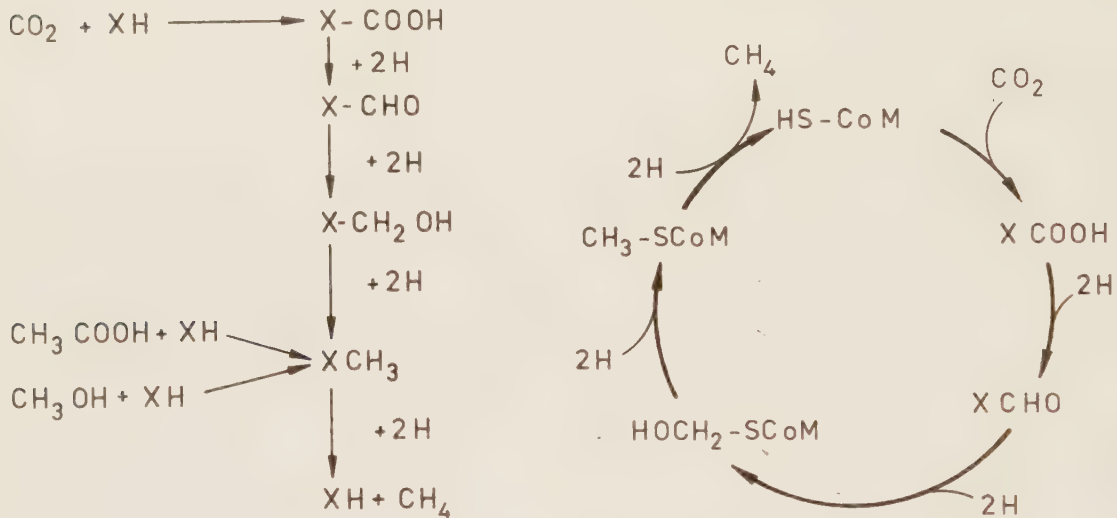


Fig 5. Reduction of CO_2 to CH_4 according to Barker, 1956 (left) and Wolfe, 1979 (right)

Apart from CoM, other coenzymes unique for methanogens have been found. One is F_{420} , an electron carrier with strong absorption at 420 nm. Another coenzyme is F_{430} - the prosthetic group of methyl CoM reductase. As F_{420} and other factors are unique for methanogens they may be useful for identification of methanogens by fluorescence microscopy or estimation of methanogenic cell mass (Doddema and Vogels 1978, Delafontaine et al 1979).

Energy metabolism

Energy conservation in methanogens may occur via substrate phosphorylation or oxidative phosphorylation. When growing on

$H_2 + CO_2$, the capacity of some methanogens to grow in the complete absence of organic compounds precludes substrate-level phosphorylation (Thauer et al 1977). When growing on acetate substrate-level phosphorylation is very unlikely since the standard free energy from acetate splitting is only -7.4 kcal/mole and ATP formation would require over 12 kcal/mole (Thauer et al 1977). It has also been shown (Zeikus et al 1975) that acetate is not to any significant extent first oxidized to $2 CO_2$ which then would be partly reduced to CH_4 . Therefore, in acetate degradation methane is formed from the methyl group.

Thus it appears likely that ATP in methanogens is generated via electron transport phosphorylation although cytochromes and quinones, essential for electron transport in other organisms, have not been found in methanogens. A scheme for ATP generation in accordance with the chemiosmotic theory has been proposed (Wolfe 1979, Smith et al 1980), Fig. 6.

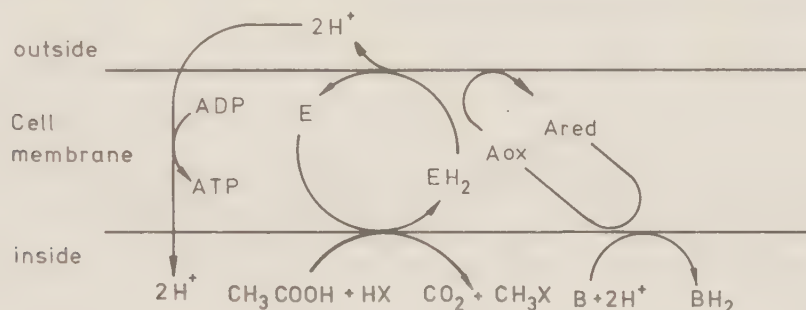


Fig 6. Proposed scheme for electron transport phosphorylation in methanogens

Morphology and composition of cell walls and lipids

Although methanogens have many physiological and biochemical features in common, they are morphologically a very diverse group of bacteria. Four main morphological groups are represented: Sarcina, rods, spheres and spirals (Zeikus 1977, Balch et al 1979). Methanosarcina does not look like the ordinary sarcina; rather it usually appears as a large, unevenly segmented coccus. Methanosarcina often clumps and forms large aggregates. Methanogenic rods (Methanobacterium, Methanobrevibacter, Methanomicrobium) may be straight or curved, short or long and filamentous, sometimes depending on cultural conditions. Coccal-shaped cells (Methanococcus, Methanogenium) may be spherical or ellipsoidal, arranged in pairs or chains. Methanospirillum consists of regularly curved rods, forming continuous spiral filaments. Flagella and fimbriae have been demonstrated on some methanogens (Doddema et al 1979).

The cell wall composition of methanogens differs considerably from that of other bacteria, e.g. no muramic acid has been found (Kandler and König 1978). Cell walls of some methanogens

contain pseudomurein containing only L-amino acids and no acetylmuramic acid. Cell walls of other methanogens contain mainly protein sub-units or heteropolysaccharides (galactosamine, neutral sugars, uronic acids) but no murein or pseudomurein.

Furthermore, the composition of lipids from methanogens is different. While typical bacteria contain saponifiable lipids the major lipids of methanogens are polyisoprenoid lipids. The neutral lipid fraction is dominated by squalene (C₃₀) whereas C₂₀ phytanyl and C₄₀ biphytanyl glycerol ethers dominate the polar fraction (Tornabene and Langworthy 1979, Tornabene et al 1979).

ENVIRONMENTAL FACTORS AND FERMENTATION PARAMETERS

Substrates for methane fermentations

A wide variety of substrates are used in or considered for methane production, see Table 2.

Table 2. Current and potential substrates for methane fermentations

1. Farm wastes
 - Swine, cattle, sheep and poultry wastes
 - Straw and other crop residues
 2. Municipal wastes
 - Municipal wastewaters
 - Sewage sludge
 - Urban solid waste
 - Algae from water treatment
 3. Wastes from food and fermentation industries
 - Brewery and distillery wastes
 - Vegetable processing wastes, e.g. starch and sugar
 - Meat packing and slaughterhouse wastes
 4. Forestry wastes
 - Wood wastes
 - Wastewaters from pulp and paper industries
 5. Energy crops
 - Crops like lucerne, sugarbeets etc.
 - Hay, grasses, leaves
 - Seaweed, kelp, water hyacinth
-

The main purpose of a digestion process may vary depending on the type of substrate. For example, in waste water treatment

the most important purpose is to reduce the COD to levels accepted for disposal in recipients and methane is only a by-product. On the other hand, in methane production from energy crops a substantial net energy yield is the main purpose.

Not all wastewaters or organic materials are suitable for methane fermentations. An important parameter is the dry matter content of the substrate. The lower limit for wastewater treatment is around 0.1 % (w/w) dry matter and the upper limit for concentrated substrates around 25 % (Frostell 1979). This makes municipal wastewaters unsuitable since the substrate concentration is too low for microbial growth and, furthermore, the heating of large volumes of water would be extremely costly. On the other hand, dry substrates such as wood residues would create technical problems with the handling and mixing of reactor contents so that the energy content may be better utilized by burning. However, current research on digester design, as discussed below, may extend applications of methane fermentation towards very dilute as well as dry substrates.

Biodegradability of substrates

Most organic polymers such as carbohydrates, lipids and proteins are regarded as biodegradable during anaerobic conditions in a digester (Hobson et al 1974) - although the degradation rates may vary considerably. The main exception are lignins which are virtually undegradable anaerobically. This may cause problems in methane production from lignocellulosic wastes such as wood, straw and manure. Cellulose and hemicellulose are by themselves readily biodegradable but when associated with the lignocellulose complex less than 50 % may be available for anaerobic degradation (Golueke 1973). Furthermore nitrogen-containing organic materials like proteins and nucleic acids may be difficult to degrade, especially when part of larger biological structures like cell walls and cell membranes (McCarty et al 1978). A way to increase biodegradability may be thermochemical or enzymatic pretreatment. This will, however, add to the costs and the heat treatment in combination with extreme pH may cause formation of toxic compounds (McCarty et al 1978).

Nutrient requirements

The bacteria participating in anaerobic digestion require sources of carbon, nitrogen, phosphorus, salts and various growth factors. The requirements of different bacterial species may be quite different, e.g. some methanogens are completely autotrophic while many fermentative bacteria need several organic growth factors.

Substrates for methane fermentations should be balanced regarding C, N, P-ratios and contain all needed salts and growth factors. A C:N-ratio of 20-30:1 is often regarded as

suitable while the phosphorous requirements are about 20 % of the nitrogen requirement (McCarty 1964, Stafford et al 1980). However, if a fermentation with cell retention is used nutrients are recycled and considerably higher C:N-ratios in the substrate acceptable. In the digestion of some materials, e.g. straw, addition of nitrogen is necessary to avoid nitrogen limitation while in animal wastes the nitrogen-levels may be too high causing ammonium-toxicity.

As for the requirement of organic growth factors like vitamins and amino acids and trace elements like Ca, Mg, K, Zn, Fe and Co little is known but it is usually assumed that these are present in sufficient quantities in most complex substrates. In pure cultures or enrichments of methanogens on defined substrates requirements for several growth factors have been demonstrated. Some methanogens, for example, need 2-methylbutyric acid and are stimulated by threonine, histidine and methionine (Bryant et al 1971). Stimulation by iron addition has been demonstrated by Speece and Engelbrecht (1964) and Patel et al (1978). Recently, Schönheit et al (1979) found a requirement for Ni, Co and Mo in Methanobacterium thermoautotrophicum. Surprisingly high levels ($\sim 5\mu\text{M}$) of nickel - which is generally not essential for growth of bacteria - are recommended. However, fermentor liquid containing H_2S in contact with stainless steel will probably provide enough nickel.

Another stimulating factor, often claimed in the literature, is the presence of inert, solid surfaces like straw, CaCO_3 and asbestos. Speece and Engelbrecht (1964) found that the rate of acetate utilization in digestors was doubled in the presence of 30 g/l finely ground asbestos.

Inoculum

Bacteria may enter a digester in two ways: either in the original inoculum or continuously via the substrate. Eventually a microflora adapted to the actual substrate and environmental conditions will develop. This may take considerable time, e.g. Hattingh et al (1967) have reported an uncompleted adaption to a new substrate after 60 days operation.

The inoculation method used in most fermentation processes - a large substrate volume and small inoculum volume - is not suitable in methane fermentations. Different groups of bacteria have different growth rates and if there is an excess of easily degraded material fermentative bacteria will produce acids faster than acetogenic and methanogenic bacteria can consume. The result will then be a falling pH and a "sour" digester. Therefore a heavy inoculum is desired and the usual method of starting small scale digestors is to fill the digester with sewage sludge and then start substrate addition at a low loading rate. This is not practical in large scale digestors since very large volumes of sewage sludge would be

required. One method could then be to use dewatered sludge (Frostell 1979). It is also possible to add increasing amounts of substrate to a digester originally filled with water (Hobson et al 1974).

Temperature

Methane production may occur in a wide range of temperatures, from almost freezing temperatures in tundra peat to over 90° C in hot springs (Zehnder 1981). The activity of methane production is low at low temperatures but, according to the theory, reaction rates should double for each 10-15° C rise in temperature. Two optimum temperature levels are generally recognized, a mesophilic level at 30-40° C and a thermophilic level at 50-60° C while the temperature maximum is around 65° C. It is often stated (Stafford et al 1980) that one set of bacteria is active in the mesophilic range and another in the thermophilic range and that considerable time is required for development of thermophilic cultures from mesophilic inocula. However, our own results (Hansson, paper IV) demonstrated a very quick transition of mesophilic cultures to thermophilic conditions indicating that the same bacteria were active in a wide temperature range.

The benefits and drawbacks of thermophilic compared to mesophilic digestion has been much discussed in the literature, e.g. in a review by Buhr and Andrews (1977). The advantages of a thermophilic process are summarized as:

- higher rates of digestion
- greater conversion of organics to gas, i.e. higher gas yields
- improved solids - liquid separation
- increased destruction of pathogenic organisms

Disadvantages of thermophilic digestion may be

- high energy requirement for heating
- larger quantities of dissolved materials in the supernatant, i.e. higher COD in effluents
- poor process stability

No doubt the amount of energy needed for heating is a major drawback requiring good insulation of digestors and heat recovery from effluents. This problem is true also for mesophilic digestors but is evidently greater in thermophilic systems. Substrate concentration is important in this context - if dilute substrates are to be heated to process temperature the energy requirement may be greater than that which the digestion can produce. In some industrial applications, however, where cheap waste heat may be available or warm wastes are to be digested the heating problem may be small.

As for the benefits of thermophilic digestion the literature

data are conflicting. Some workers have demonstrated high rates and yields in thermophilic methane production from municipal refuse (Pfeffer 1974) or manure (Varel et al 1977). On the other hand, several other workers found no improvement or even lower gas production at thermophilic compared to mesophilic conditions (Maly and Fadrus 1971, Hansson, papers IV, V). There may be several reasons for these discrepancies: improper selection or acclimatization of inocula, differences in feed composition or inhibition by unknown compounds formed during thermophilic conditions. Care must also be taken when comparing rates of fermentation with different cell densities. The temperature should affect the specific activity of bacteria while the gross fermentation rate is also affected by the number of bacteria.

pH, volatile fatty acids, alkalinity

Methane fermentations in complex cultures proceed quite well in the pH range 6.6 - 7.6 with an optimum at pH 7.0 - 7.2. The activity drops rapidly below pH 6.2 (McCarty 1964) although methane is produced in acid peat bogs. Pure cultures of methanogens are physiologically most active in the pH range 6.7 - 8.0 (Wolfe 1979).

In normally working methane fermentations pH is controlled by the buffering action of various acid-base systems. The major buffering system is the CO_2 /bicarbonate-equilibrium:



Thus, pH is determined by the bicarbonate concentration and the CO_2 partial pressure (McCarty 1964). Other acid-base systems are the orthophosphoric ($\text{H}_2\text{PO}_4^{2-}/\text{HPO}_4^-$), the ammonia-system ($\text{NH}_4^+/\text{NH}_3$), the hydrosulphuric ($\text{H}_2\text{S}/\text{HS}^-$) and volatile fatty acid equilibria (e.g. $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$). However, in most cases the phosphates and sulfides are present in concentrations that are too low to be significant. Furthermore, since the pK_a -values of the ammonium and VFA-systems are 9.3 and around 4.8, respectively, the buffering action of these systems in normal pH ranges of methane fermentation is small (Capri and Marais 1975). Thus, the buffering capacity, usually expressed as alkalinity, is mainly due to the bicarbonate system.

If a methane fermentation is disturbed, e.g. by overloading, presence of toxic materials or cultural imbalance during start-up, methanogenic bacteria will not consume acetate and hydrogen as efficiently as before. This will lead to an inhibition of acetogenic bacteria, increased VFA-levels and a higher proportion of reduced acids as discussed above. Finally the buffering capacity is consumed and the pH will drop. Obviously the remedy should be to remove the cause of disturbance but to overcome a temporary critical period the addition of an alkali like lime or sodium bicarbonate may be necessary (McCarty 1964). Apart from the effects on pH, high levels of volatile fatty acids may be toxic, as discussed below.

Redox potential

Highly reduced environments are essential in methane fermentations. Redox potentials may be measured by means of an inert electrode (platinum or gold), a reference electrode (usually a calomel electrode) and a conventional mV-meter (Blanc and Molof 1973). Literature data on potentials are often expressed in two ways, as E_c or E_h :

$$E_c = E_h - E_{\text{calomel}}$$

where E_c is the measured potential between the inert and reference electrodes, E_h is the potential against the standard hydrogen electrode and E_{calomel} is the potential of the calomel electrode against the standard hydrogen electrode (= +244 mV at 25°C).

Optimum methane production occurs at electrode potentials (E_c) between -510 and -530 mV and proceeds reasonably well in the range -450 to -500 mV (Blanc and Molof 1973 and others). Above -360 mV methane production seems to be completely inhibited.

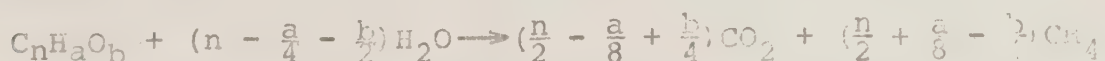
It should be noted that there are several difficulties in measuring redox potentials. The platinum electrode is not inert and many redox processes are very slow in reaching equilibrium in the solution and with the electrode (Zehnder 1978). Furthermore, the measured potential in complex solutions are probably "mixed electrode potentials" where several redox levels occur in the same environment because they do not couple with one another (Blanc and Molof 1973, Zehnder 1978). It is also possible that the "mean" redox potential in the liquid phase is different from the potential in the central parts of clumps and particles in complex media.

Gas yields

The yield of methane is one of the most important parameters in methane fermentations, especially in applications where energy production is the main purpose. Some major factors affecting the yields of CH_4 and CO_2 in methane fermentation are:

- chemical composition of the substrate
- biodegradability of complex substrate components
- retention time of bacteria and substrate
- amount of substrate assimilated by bacteria

Theoretical gas yield may be predicted by the formula of Buswell and Mueller (1952):



Methane production may also be predicted from an estimate

COD (McCarty 1964). One mole of CH_4 is equivalent to 2 moles of O_2



and therefore 1 kg COD-reduction is equivalent to $0.35 \text{ m}^3 \text{ CH}_4$ (STP). From these data theoretical gas yields, gas composition and COD/VS-ratio have been calculated and compiled in Table 3.

Table 3. Gas yield, gas composition and COD/VS-ratio in methane fermentation of pure organic compounds

Material	Gas yield m^3/kg		Gas composition % CH_4 (v/v)	COD/VS $\text{kg O}_2/\text{kg}$
	CH_4	$\text{CH}_4 + \text{CO}_2$		
Carbohydrate	0.37	0.75	50	1.06
Fat	1.04	1.44	72	2.97
Protein	0.49	0.98	50	1.40

All the CO_2 produced will not be released as gas. Some CO_2 will dissolve and leave the fermentor in the liquid phase² as bicarbonate. Therefore, in most fermentations on complex substrates gas composition is 60-70 % CH_4 and 30-40 % CO_2 (Meynell 1976). The biogas will also contain small amounts of H_2S , CO and N_2 depending on substrate composition. The content of water vapor is around 4 % at 30°C and 20 % at 60°C .

Gas yields close to the theoretical values are usually obtained on easily degraded substrates. Only small amounts of substrates are assimilated by bacteria - the resulting small production of sludge is one of the advantages of anaerobic wastewater treatment. On complex media, however, the biodegradability may be poor, as discussed above, and gas yields low. To increase gas yields different approaches are possible:

- increasing sludge retention time (SRT), i.e. contact time between bacteria and substrate. Much progress has been made during the last two decades to improve fermentor design for this purpose
- pretreatment to improve biodegradability. Fermentations may be carried out in two steps with a hydrolysis reactor as the first step
- optimizing "biological" parameters like quality of inocula, environmental factors, removal of toxic compounds and use of stimulating agents

It should be emphasized that the gas yield is only one part of the net energy production of the system and energy requirements for heating, mixing etc. may be equally important.

Inhibitors

Many organic and inorganic compounds are toxic to methane fermentations. Toxicity is very much a matter of concentration. Most compounds are stimulatory at low concentrations and toxic at higher concentrations and toxic concentrations may range from under 1 mg/l to several thousand mg/l. Also acclimatization to higher levels of toxic compounds may occur. Although many bacteria in methane-producing cultures may be sensitive to toxic materials, methanogens are usually regarded as the most sensitive group since toxicity usually causes a fall in methane production accompanied with increase in VFA-concentration.

Some alkali and alkaline earth-metal salts are stimulatory, mildly or strongly inhibitory depending on concentration, Table 4 (McCarty 1964). Certain combinations of these metals have synergistic effects while other combinations (e.g. Na and K) have antagonistic effects (Kugelman and Chin 1971).

Table 4. Stimulatory and inhibitory concentrations (mg/l) of some metals, ammonia and sulfide

Material	Stimulatory	Mild inhibition	Strong inhibition
Na	100-200	3500-5500	8000
Ka	200-400	2500-4500	12000
Ca	100-200	2500-4500	8000
Mg	75-150	1000-1500	3000
NH ₃	50	-	150
NH ₄ ⁺	200-1000	1500-3000	3000
S ²⁻	50-100	100-200	200

Low, but soluble, concentrations of heavy metals like Cu, Zn, Ni, Cr are toxic in anaerobic digestion although many metals are required in trace amounts. Fe- and Al-salts are not toxic due to their low solubility (McCarty 1964). Toxicity levels are in the range 10^{-3} to 10^{-4} M. Considerably higher levels of heavy metals in substrates can be tolerated since heavy metals take part in complex-type reactions like precipitation

by sulfides and sequestering by ammonia or bacterial mucopolysaccharides. Toxicity by heavy metals can be completely eliminated by precipitation as sulfides. If enough sulfide is not present, FeSO_4 may be added as a sulfur-source (Kugelman and Chin 1971). However, sulfide itself is also toxic. Sulfides can enter with the substrate or be formed by a reduction of sulfates or other sulfur-containing compounds. Sulfides are present in an insoluble form depending on the amount of cations and as soluble sulfides and gaseous H_2S depending on the pH and total gas production (McCarty 1964). Inhibition starts at 100-200 mg/l as soluble sulfide although considerable acclimatization to higher levels of sulfide has been reported. Toxic levels of sulfide may be reduced by addition of ferrous chloride or by gas scrubbing.

Some substrates for methane production, e.g. manure, may contain high levels of ammonia and more ammonia may be formed during decomposition of N-containing compounds. Ammonia is present as NH_4^+ and NH_3 in equilibrium with $\text{pK}_a = 9.3$. NH_3 is considerably more toxic than NH_4^+ (Table 4) and toxicity starts at total ammonia concentrations of between 1500 and 3000 mg/l and $\text{pH} > 7.4$. At concentrations above 3000 mg/l NH_4^+ itself is toxic regardless of pH (McCarty 1964). However, van Velsen and Lettinga (1979) and others report on acclimatization of cultures to higher ammonia levels (up to 5 g/l) and higher pH (7.5-8.0).

Another source of inhibition may be high levels of volatile fatty acids as a result of the culture being disturbed. Apart from the effect on pH, which can be corrected by natural buffer systems or alkali additions, high levels of dissociated acids remain and some workers have found that acid levels around 2000 mg/l are toxic to methanogens but not to acid-formers. However, Kugelman and Chin (1971) demonstrated that VFA are not toxic to methanogens at concentrations up to 6000 mg/l. Therefore, alkali addition during unbalanced digestion should be safe as long as toxic amounts of cations are not added.

Some examples of other materials toxic to methane fermentations are: organic solvents, alcohols, long-chain fatty acids, pesticides, cyanide and chlorinated hydrocarbons like chloroform (Meynell 1976, McCarty 1964). Oxidants like O_2 , NO_3^- and SO_4^{2-} may influence the redox potential and disturb the balance between different groups of bacteria and possibly have direct toxic effects.

REACTOR DESIGN

A great deal of information on reactor design is available in the literature, for reviews and further references see Stafford et al (1980) and Meynell (1976). Only a brief review of some important reactors is given here.

Batch digestors

These types of simple, unheated and non-mixed digestors were much used during World War II. Wastes to be treated, like straw, leaves and dung were placed in a tank, pit, trench or bag together with seed material. The digestor was sealed and the waste material digested. After the completed gas production the contents were removed.

A new approach to batch digestors has been taken by W J Jewell at Cornell University and others. A simple batch digestor for dry substrate applications has been studied (Jewell 1979). This would operate with high solid substrates like straw and manure with 25-30 % TS and 100-300 days retention time. Several advantages are likely, for example the exothermic energy produced during digestion may be enough for heating the digestor to mesophilic temperatures.

Continuous fermentors without cell-recycling

A wide range of fermentors are in use or have been described. There are non-mixed plug-flow, and completely mixed fermentors; single stage or multistage; heated or unheated; continuous or batch-fed and designs varying from simple holes in the ground with rubber-covers to highly instrumented stainless steel reactors.

An example of a simple reactor is the Chinese-type digestor (Fig. 7) intended to supply energy for cooking and heating in rural areas. This is a 6-8 m³ digestor built of bricks and concrete and fed daily with animal and human wastes. China is reported to have 7 million digestors of this type in operation (Nyns et al 1979). A similar type of digestor are the Gobar gas plants being operated in India.

"High-rate digestors" are the continuously working, completely or intermittently mixed and temperature controlled fermentors of the type usually employed at sewage works (Fig. 7). They are usually cylindrical or egg-shaped and built from steel or concrete. Mixing is provided by mechanical stirring, by recirculation of liquid or by gas recirculation. The high-rate digestor is usually employed for the digestion of concentrated substrates although difficulties in mixing viscous liquids sets a limit to TS-values. A very large number of pilot and full-scale high-rate digestors are in operation all over the world.

Fermentors with cell-retention

In a completely mixed system the liquid retention time (LRT) and solids retention time (SRT) are the same. By retaining solids - in which most bacteria are found - the fermentor will operate with higher cell densities and greater efficiency.

Also, if the substrate contains insoluble complex materials the time for degradation of these compounds will increase. This could be done in a conventional high-rate digester. For example, if mixing is intermittent the fermentor contents will settle during periods without mixing and a fraction of the thinner liquid may be withdrawn from the fermentor while thicker liquid is retained. However, reactors with cell retention have mostly been applied to anaerobic waste water treatment so far. Some fermentor-types are shown in Fig. 7.

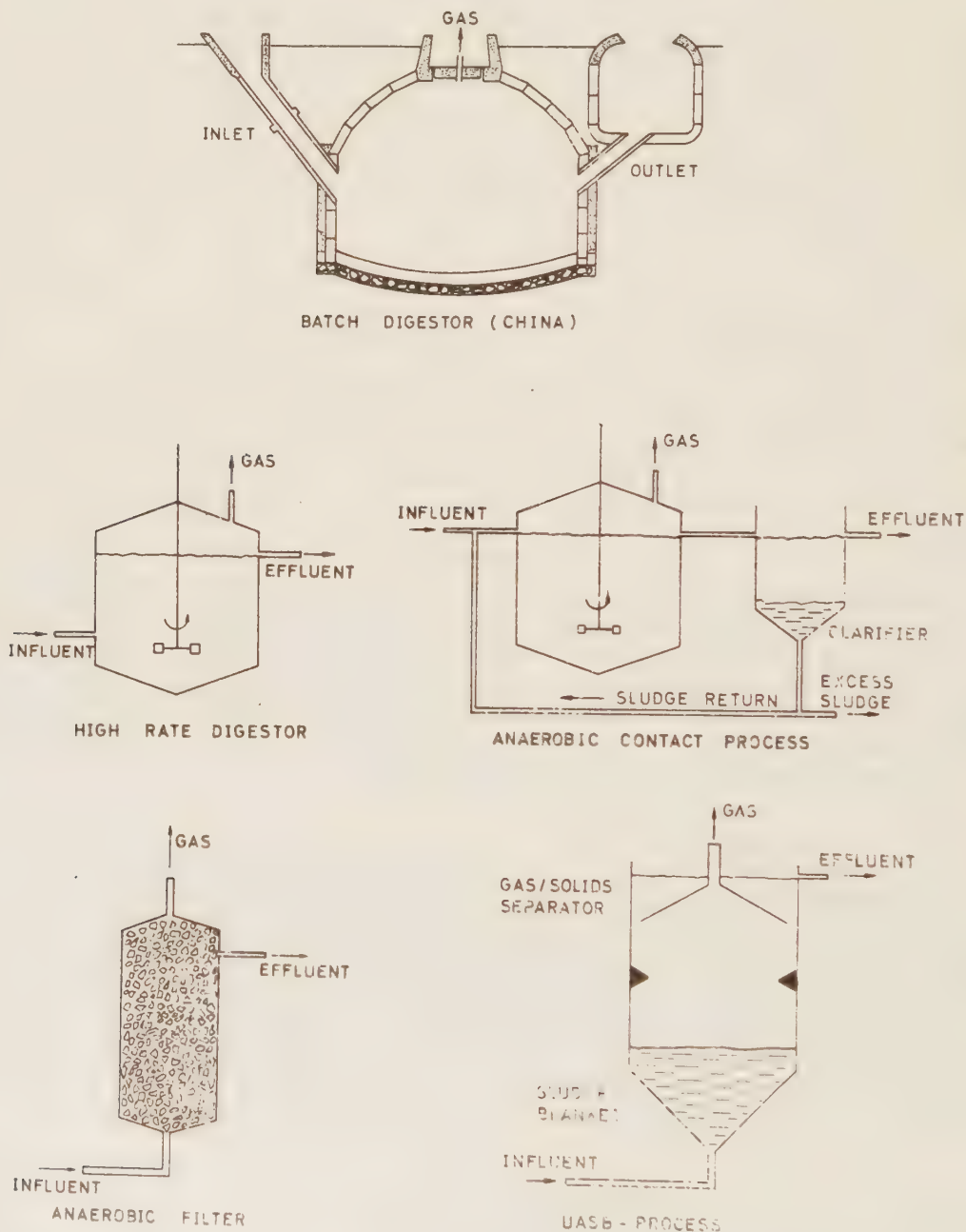


Fig 7. Some examples of reactor design in anaerobic digestion and waste-water treatment

The anaerobic contact process is an analogy of the aerobic activated sludge process. Solids are separated in a clarifier and returned to the fermentor. The settling of solids is a major problem since gas bubbles adhering to the sludge will impair the sedimentation.

Another process is the anaerobic filter, an analogy of the aerobic trickling filter. The anaerobic filter consists of a tank containing stone granules, ceramic rings or plastic pieces of various shapes and sizes. Thus a large surface area is created with an attached bacterial film.

The UASB- (Up-flow anaerobic sludge blanket) process was developed by Lettinga and co-workers in the Netherlands (Lettinga et al 1979). It utilizes a blanket of a granular type of anaerobic sludge with very good settling characteristics. Wastewater is passed through the blanket where organics are degraded. The reactors are equipped with gas solid separators to separate gas from dispersed sludge particles.

The UASB-process is an example of a fluidized bed system. These processes may prove valuable when it comes to treating very dilute wastewaters, maybe even municipal sewage waters. Switzenbaum and Jewell (1980) reported on the "anaerobic attached film expanded bed reactor" (AAFEB), in which bacteria are attached to small particles in a fluidized bed. Treatment of low-strength wastewaters (COD <600 mg/l) at low temperatures (10-20°C) was successful, probably due to a large biomass concentration, up to 30 g/l.

KINETICS OF METHANE FERMENTATIONS

Methods and problems in kinetic studies

Knowledge concerning anaerobic fermentation kinetics is valuable in order to understand the behaviour of methane fermentations, to predict performance and possible treatment results and to evaluate design and operation of different anaerobic digestion systems.

Bacteria participating in methane fermentations may be isolated and studied in pure culture employing conventional kinetic models of pure culture growth. Apart from the amount of work involved and the technical difficulties in cultivating methanogens and acetogens, the question is whether the bacteria carry out the same reactions as they do in complex cultures. As discussed above, many bacteria are closely interrelated, for example by interspecies hydrogen transfer. A fermentative bacterium may have a quite different fermentative pattern in pure culture than in mixed culture.

Kinetics is often studied in complex cultures, either enrichment cultures, e.g. on fatty acids or glucose, or in complete ecosystems like rumen or sewage sludge fermentations. Often kinetic models of pure culture growth in steady state, such as the Monod model, are used to describe the complex process of anaerobic digestion although the validity of this approach may be questioned (Hobson et al 1974, Chen and Hashimoto 1978). In this method the kinetic model is applied to the rate-limiting step which governs the over-all process rate.

The study of kinetics in complex cultures is associated with several problems including the following:

- To measure various specific activities it is necessary to measure the cell mass of the bacteria carrying out that particular activity. This is extremely difficult in a mixed bacterial system, often containing particulate matter.
- The rate-limiting reaction has to be established. In methane-production from soluble substrates the methanogenic step is usually regarded as rate-limiting (Ghosh and Pohland 1974), i.e. acetate degradation by methanogens since methane-production from $H_2 + CO_2$ is much faster. However, recent information on acetogenic bacteria indicates that bacteria degrading propionic and butyric acid are considerably slower having generation times of 108-160 hours compared to 24-72 for acetate-degrading methanogens. On the other hand, butyrate and propionate are only minor intermediates in a working anaerobic digestion.

In methane production from complex materials the initial hydrolytic steps are probably rate-limiting. Cellulose degradation is suggested as the rate-limiting step in the digestion of municipal solid waste (Pfeffer 1974) and cattle waste (Hobson et al 1974) while long-chain and short-chain fatty acids may be rate-limiting substrates in sewage sludge digestion (McCarty 1971).

- While steady state may be easily obtained in pure culture of fast-growing bacteria, complex slow-growing cultures on complex substrates require several months to reach steady-state - if they ever do (Hattingh et al 1967). Furthermore, in practical applications there are usually fluctuations in feed rate and composition.

Kinetics of methane production from soluble substrates

The rate-limiting reaction in methane fermentation of dissolved organic matter is usually assumed to be



carried out by methanogens. In a completely mixed reactor at steady state without cell recycle the growth rate of micro-organisms may be described by

$$\frac{dX}{dt} = Y \cdot \frac{dS}{dt} - k_d \cdot X \quad (1)$$

and the relation between specific growth rate and limiting substrate concentration by a Monod equation:

$$\mu = \frac{\mu_{\max} \cdot S}{K_s + S} \quad (2)$$

where X = cell mass (in this case of methanogens degrading acetate)

S = concentration of limiting substrate (in this case acetate)

Y = yield coefficient

k_d = decay coefficient

μ = specific growth rate

$\mu_{\max}$ = maximum specific growth rate

K_s = half velocity coefficient

In steady state

$$\mu = D = \frac{1}{LRT} = \frac{1}{SRT} \quad (3)$$

where D = dilution rate

LRT = liquid retention time

SRT = solids retention time

If the acetate concentration S in steady states at different μ are determined, $\mu_{\max}$ and K_s can be calculated from eqn (2). If the cell mass concentrations X in the steady states are also determined Y and k_d can be calculated from eqn (1). Some literature data on these kinetic parameters is available, for example, from work by Lawrence and McCarty (1969) on acetate enrichments at 35°C:

$$\begin{aligned} \mu_{\max} &= 0.5 \text{ days}^{-1} \\ K_s &= 207 \text{ mg/l} \\ Y &= 0.040 \text{ mg/mg} \\ k_d &= 0.019 \text{ days}^{-1} \end{aligned}$$

Since the generation time $g = \frac{\ln 2}{\mu}$ it follows that the minimum generation time

$$g_{\min} = \frac{\ln 2}{\mu_{\max}} = 1.4 \text{ days} \quad (4)$$

This agrees well with pure culture studies. Methanosarcina barkeri grows with generation times of 24-72 hours.

From the practical point of view the required solids retention time (SRT), the effluent waste concentration and the treatment efficiency (E) are important parameters. E is defined as

$$E = \frac{S_0 - S}{S_0} \quad (5)$$

where S_0 = influent and S effluent organic concentrations. The relationship between E, S and SRT may be calculated from eqns (2) and (3) and known kinetic parameters. One example is illustrated in Fig. 8 (Lawrence and McCarty 1969):

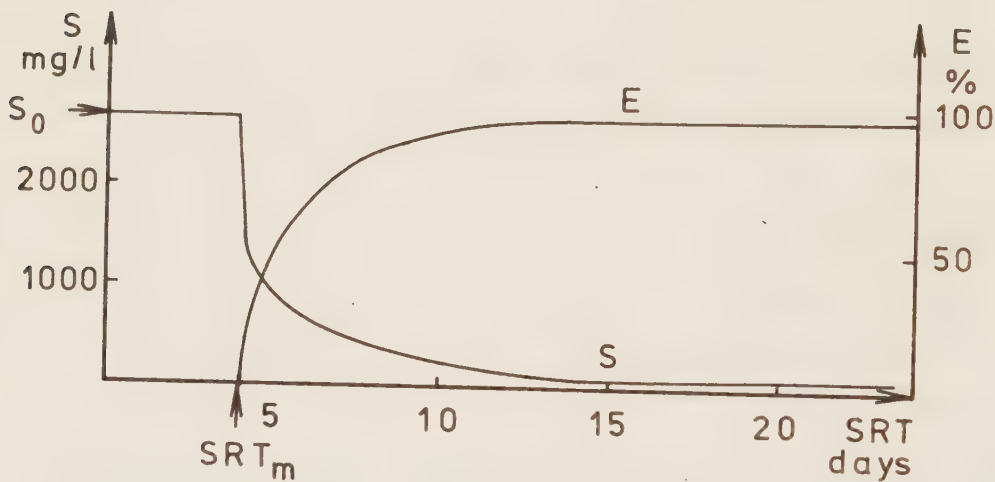


Fig 8. The influence of solids retention time (SRT) on treatment efficiency (E) and effluent organic concentration (S). Based on kinetic data from Lawrence and McCarty (1969)

While the minimum solids retention time ($SRT_m = \mu_{max}^{-1}$) is usually 2-5 days, depending on the substrate, temperature etc., the actual retention time should be at least three times greater to ensure good process stability and treatment efficiency.

Kinetics of methane production from complex materials

Also when studying the kinetics of methane fermentation of

complex substrates such as municipal and animal residues the rate-limiting step approach may be utilized, although it is very difficult to determine what the rate-limiting step is and analyze the concentration of rate-limiting substrates. However, McCarty (1971) suggested a kinetic model based on Michaelis-Menten type kinetics and the assumption that cellular yield of microorganisms is a function of reaction energetics. When the model is applied to a complex substrate containing carbohydrate, protein and fat the concentrations of various compounds as a function of cell retention time may be predicted. According to this model proteins and carbohydrates should be degraded considerably faster than long and short-chain fatty acids. Experimental data from methane fermentation of primary sewage sludge supported this model and suggested the degradation of fatty acids as rate-limiting reactions (O'Rourke 1968).

Andrews and Graef (1971) suggested a dynamic model where unionized acids are considered as rate-limiting substrates as well as inhibiting agents and therefore an inhibition function has been incorporated into the Monod function.

Another approach is to use kinetic models based on total substrate concentrations such as volatile solids (VS) or chemical oxygen demand (COD) rather than limiting substrate concentrations. For example, Pfeffer (1974) used a standard first-order rate-expression when studying methane production from domestic refuse.

$$\frac{S_0 - S}{S} = K \cdot \theta$$

where S_0 = initial concentration of organic solids

S = concentration of organic solids in the reactor

θ = liquid retention time

K = rate constant

Chen and Hashimoto (1978) suggested a Generalized Substrate Utilization Kinetic Model given by the equation

$$\frac{\mu}{\mu_{\max}} = \frac{S/S_0}{K + (1-K)S/S_0}$$

where μ = specific growth rate

$\mu_{\max}$ = maximum specific growth rate

S = biodegradable effluent substrate concentration

S_0 = biodegradable influent substrate concentration

K = relative substrate-microorganism binding constant

This model is applicable to pure and mixed cultures in simple as well as complex substrates. This model was used to predict parameters like total gas yields from sewage sludge, municipal

refuse and animal wastes, volumetric gas yields, $\mu_{\max}$ and K and their variation with temperature, loading rate etc. The agreement with experimental results was good.

Kinetics of methane production with cell recycle

The development of mathematical expressions for the kinetics of methane-producing fermentors with or without cell recycle is available in the literature, e.g. in a review by Kirsch and Sykes (1971) and by Pohland and Hudson (1976). When cell recycle is used, a recycle factor r is employed defined as the ratio of biological solids concentration in the reactor and the concentration in the effluent. The expressions for specific growth rate and cell concentration in a continuous, completely mixed reactor will then be:

$$\mu = \text{SRT} = r \cdot \text{LRT}$$

$$X = \frac{Y (S_o - S) r}{1 + r \cdot k_d \cdot \text{LRT}}$$

Thus, cell recycling will increase the cell density in the reactor permitting short liquid retention times.

When cell recycling is used a considerable increase in the organic loading rate is possible. Frostell (1979) used the expression

$$\text{OL} = \frac{\mu_{\max} \cdot S_o^2 \cdot r}{(K_s + S_o) f}$$

where OL = organic load

$$f = \text{safety factor} = \text{SRT} / \text{SRT}_{\min}$$

When treating dilute waste-waters (COD < 10,000 mg/l) sludge retention is necessary to achieve realistic loading rates. Sludge retention is also recommended for high-strength wastewaters, (COD > 100,000 mg/l).

SUMMARY AND DISCUSSION OF RESEARCH PAPERS

	page
END PRODUCT INHIBITION IN METHANE FERMENTATIONS	36
1. Literature background	36
2. Fermentation methods	38
3. Initial studies	40
4. Effects of $p\text{CO}_2$ in mesophilic methane fermentations	42
4.1 Effects of $p\text{CO}_2$ on glucose-fermenting bacteria	42
4.2 Effects of $p\text{CO}_2$ on propionate- and butyrate-oxidizing acetogens	43
4.3 Effects of $p\text{CO}_2$ on acetate degradation by methanogens	45
4.4 Effects of $p\text{CO}_2$ on methane yields	48
5. Effects of $p\text{CO}_2$ in thermophilic methane fermentations	49
6. Mechanism for CO_2 -effects	49
7. Effects of $p\text{CH}_4$ in mesophilic methane fermentations	51
8. Practical approaches to obtain low $p\text{CO}_2$	51
 THERMOPHILIC METHANE FERMENTATION	 53
1. Introduction	53
2. Adaption of cultures to thermophilic methane production	54
3. Methane production at extreme temperatures	56
 METHANE PRODUCTION FROM GREEN MACRO-ALGAE	 57
1. Background	57
2. Anaerobic digestion of green macro-algae	59
2.1 Inocula and start-up procedure	59
2.2 Retention times and loading-rates	60
2.3 Gas yields and VS-reduction in mesophilic fermentation	60
2.4 Thermophilic fermentation	62
 CONCLUSIONS	 63

END PRODUCT INHIBITION IN METHANE FERMENTATIONS

1. Literature background

End product inhibition is well-known in many fermentation processes and may also be expected in methane fermentations. For example, hydrogen - although not an end product of the complete ecosystem - is an end product of several fermentative and acetogenic bacterial reactions. The hydrogen partial pressure has recently been found to strongly affect the fermentative and acetogenic activities as discussed in the literature review above. Furthermore carbon dioxide is produced by many fermentative and acetogenic bacteria while methane and carbon dioxide are end products of methanogens. However, literature data on end product inhibition by CO_2 and CH_4 is scarce, especially regarding the effects on specific groups of bacteria, and the results are often conflicting.

One method of studying end product inhibition by CO_2 and CH_4 in methane fermentations is to conduct the fermentation at a reduced pressure thus resulting in low $p\text{CO}_2/p\text{CH}_4$ which in turn should give improved methane production. If high gas pressure is imposed on methane fermentations, e.g. by progressive gas evolution, high $p\text{CO}_2/p\text{CH}_4$ should impair the methane production. Buswell (1932), using dextrose as a substrate, carried out digestion at 680 atm (the actual $p\text{CO}_2$ and $p\text{CH}_4$ unknown) and reported almost complete inhibition, probably due to acid inhibition (pH 4.0). In large and deep digestors the hydrostatic pressure will increase $p\text{CO}_2$ and $p\text{CH}_4$ in gas bubbles which should increase any CO_2 - or CH_4 -inhibition. Digestion rate as a function of digester depth has been studied by Schaetzle (1924), Sierp (1927) and others but the results are conflicting. Improved digestion, inhibited digestion as well as no effect due to depth were reported. Bloodgood and Anderson (1963) studied effects of 0.3 - 2.0 atm gas pressure in continuous sludge digestion. The volatile solids reduction was the same in the 0.7 - 2.0 atm interval

but reduced by 10 % in the 0.3 atm digester. However, pH was not controlled and had increased to 7.8 - 7.9 in the 0.3 atm digester. Mangel et al (1980) investigated methane production from oat straw in pressurized fermentors at 0 - 8 atm over-pressure. Gas production decreased with increasing pressure but the percentage of methane increased and therefore the methane production was constant in the interval 0 - 4 atm. At 8 atm methane production was reduced but some gas evolution still continued at 16 atm. No details concerning pH or fatty acid levels were reported. Methane production from $H_2 + CO_2$ at elevated pressures (30 atm) has been investigated by Wise et al (1978). After some adaption of the culture methane production was comparable to production at atmospheric pressure. Finney and Evans (1975) and Finney et al (1976) reported fast methane production from both acetate and glucose in a low-pressure fermentor with $pCO_2 = 0.2$ atm and pH 6.8 - 7.2. Minimum doubling times of 0.28 days for methanogens and loading rates of 31 g VS/l·d were reported.

Another method of studying the effects of pCO_2 and pCH_4 on methane fermentations is to control the gas phase by introducing CO_2 , CH_4 or an inert gas in the fermentors. It is then necessary to use a well-mixed system to ensure good contact between the gas- and liquid phases. Accurate control of pH and redox potential is also essential. Keefer and Kratz (1932) studied how the bubbling of various gases through bottles with sewage sludge affects methane production. Sludges treated with N_2 or CH_4 digested most rapidly but also the bubbling of CO_2 , sludge gas, air, O_2 and no gas bubbling at all worked well. No inhibition was noted when pure O_2 was introduced at 0.8 litre/h in 1 litre sludge. Hartz and Kountz (1966) investigated effects of CO_2 and N_2 on anaerobic digestion of fish food in experiments where gas mixing with external CO_2 or N_2 was compared to mechanical mixing. Both gas-mixed fermentors showed decreased VS-reduction and increased redox potential compared to the mechanically mixed fermentor. pH was not controlled which resulted in pH variations from 8.0 to 5.9 in the N_2 -

digestor. Opperman et al (1957) demonstrated inhibiting effects of CO_2 in rumen cultures utilizing acetate, although pH was not controlled. Initial sparging with CO_2 delayed acetate degradation and continuous CO_2 -sparging during 24 hours reduced acetate utilization by 40 %. In our own work (Hansson 1979) oxygen-free N_2 , CO_2 or CH_4 was sparged through pH-controlled fermentors with continuous methane production from glucose. Methane production decreased and acetic acid accumulated in CO_2 -fermentors compared to N_2 -fermentors.

The results in the literature regarding end product inhibition are conflicting. In some cases the noted inhibiting effects are probably due to insufficient control of the experiments rather than CO_2 - or CH_4 -inhibition. However, it seems clear that CO_2 has inhibiting effects on some methanogens, except when grown on $\text{H}_2 + \text{CO}_2$, although the degree of inhibition at different pCO_2 is not known. No reports regarding inhibition by CH_4 have been found.

2. Fermentation methods

Accurate control of fermentation parameters is essential when studying end product inhibition in methane fermentations, as demonstrated by the varying, and partly conflicting, results in the literature. For example, any effects of CO_2 or CH_4 may be difficult to detect if pH is not controlled. The degree of end product inhibition may depend on the type of substrate. Changes in gas phase composition should be avoided and good contact between the gas and liquid phases established.

In this work end product inhibition studies were carried out in batch and continuous cultivations in fermentors with controlled temperature, pH, mixing and gas phase composition. Control of the gas phase was achieved by rapid mixing combined with sparging with oxygen-free N_2 , CO_2 , CH_4 or combinations of these gases. In this way any combination of $\text{pCO}_2 = 0 - 1$ atm and $\text{pCH}_4 = 0 - 1$ atm was possible. No adverse effects of the rapid

mixing was found (III). Another approach would have been to use low or high total pressures. However, since $p\text{CO}_2 \sim p\text{CH}_4 \sim 0.5 P_{\text{tot}}$ fermentations in almost a vacuum and 2 atm would be required to cover the range $p\text{CO}_2 = p\text{CH}_4 = 0 - 1$ atm. It was thought that such systems would create technical problems such as air leakage and very high vapour content when using low pressures.

Another important question was whether to use pure and defined cultures or unknown enrichments. Normally, kinetic studies in fermentations are much easier in pure culture, for example since it is possible to measure the cell mass. However, for several reasons it was decided to use enrichment cultures. Firstly, a methane-producing culture is an ecosystem of inter-related bacteria which may react differently in pure culture. Secondly, some of the participating bacteria are difficult, maybe impossible, to cultivate in pure culture and, thirdly, it is not certainly known what reactions are rate-limiting. Therefore a mesophilic, continuous glucose-enrichment, originally from sewage sludge and then cultivated on glucose for about 3 years, was used as inoculum in the end product inhibition experiments. In each experiment a series of fermentors were run in parallel and inoculated simultaneously with the same culture to avoid problems of slight variations in inoculum culture conditions. Furthermore, in each set of fermentations a reference fermentation with N_2 -sparging was always included to make results from different sets of fermentations comparable.

To study the effects of $p\text{CO}_2$ and $p\text{CH}_4$ on the kinetics of fermentative, acetogenic and methanogenic bacteria, substrates based on glucose, mixed short chain fatty acids or acetic acid alone were used. This means that only the later stages, and not kinetics of the degradation of complex materials, were studied. On the other hand, the degradation of acetate, propionate and butyrate are reactions common to all methane fermentations, also on complex substrates. Apart from inoculum preparation and some initial studies (I) on end

product inhibition, which were made in continuous culture, fermentations were carried out as batch cultivations. The activity of the various bacterial groups were measured as rate of substrate removal and corresponding methane formation. This method was found to be reliable and comparatively fast, one fermentation experiment being carried out in 3 - 12 days, depending on $p\text{CO}_2$. One alternative would be to determine kinetic constants by measuring steady state concentrations of the rate-limiting substrate as a function of retention time in continuous culture. This method was tested (unpublished data) but resulted in considerably longer fermentation experiments and difficulties in maintaining steady state.

3. Initial studies (I)

Some preliminary tests were carried out to qualitatively establish whether CO_2 and CH_4 were inhibitory to methane production. This work was done with the glucose enrichment culture in continuous cultivation on glucose. By rapidly switching between N_2 - and CO_2 -sparging, methane production and fatty acid levels were measured in fermentations at $p\text{CO}_2 = 0.05 \text{ atm}$ and $p\text{CO}_2 = 0.95 \text{ atm}$. One example is shown in Fig. 1.

The rapid response in methane production and acetate levels on changes in $p\text{CO}_2$ clearly suggested that CO_2 affected acetate utilization. The rate of acetate utilization was reduced by 46 % during CO_2 -sparging in this experiment. However, since the culture was substrate-limited during N_2 -sparging, the real difference in acetate utilization capacity during N_2 - and CO_2 -sparging could not be determined.

In several cases during CO_2 -sparging the acetate concentration increased to 2 - 3 g/l and, due to NaOH-titration, Na-concentration rose to 3 - 6 g/l, in one case even 9 g/l. The acetate level should not be toxic (Kagelman and Chin 1971). McCarty (1964) states that Na-concentrations of 3.5 - 5.5 g/l

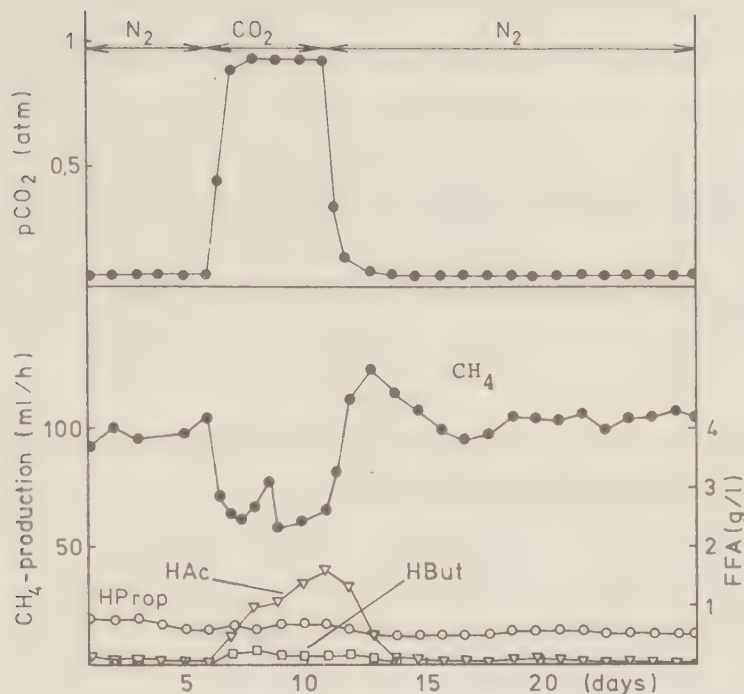


Fig 1. Effects of rapid changes of $p\text{CO}_2$ on methane production and fatty acid levels in continuous methane production from glucose

are moderately inhibitory and 8 g/l strongly inhibitory. However, in this work, rates of methane production and acetate utilization immediately returned to values normal at low $p\text{CO}_2$ as a result of change from CO_2 - to N_2 -sparging despite the high levels of Na and HAc. Therefore the inhibition was not due to Na- or HAc-toxicity and, evidently, methane-producing cultures may adapt to high levels of sodium.

Moreover, methane production at high levels (0.85 - 0.90 atm) of CH_4 was studied. In this case the high $p\text{CH}_4$ was achieved by a gas recirculation system in which CO_2 was absorbed in alkali and the resulting CH_4 returned to the fermentor. No decrease in the methane production rate and no accumulation of acetate was found during the high $p\text{CH}_4$ -experiments.

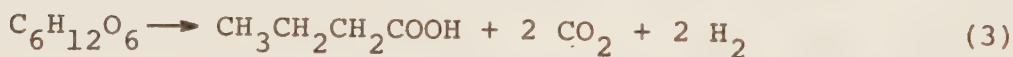
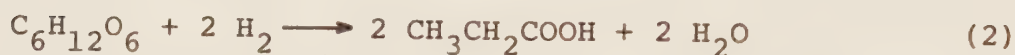
Consequently, these preliminary studies demonstrated inhibiting effects of CO_2 but no inhibition due to CH_4 . However,

further research was necessary to establish the degree of inhibition as a function of gas phase composition.

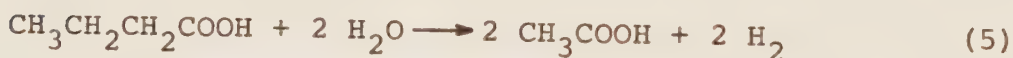
4. Effects of $p\text{CO}_2$ in mesophilic methane fermentations

Three main groups of bacteria participate in methane production from glucose:

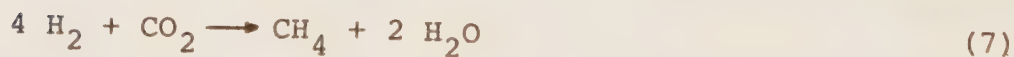
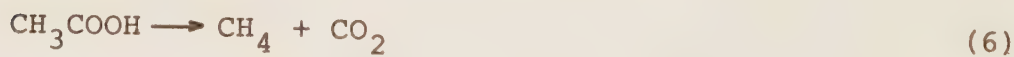
I Acetic-, propionic- and butyric acid production from glucose by fermentative bacteria:



II Oxidation of propionic acid and butyric acid by obligate proton-reducing acetogenic bacteria:



III Methane production from acetic acid and H_2/CO_2 by methanogens:



In this work effects of $p\text{CO}_2$ on the kinetics of glucose-, propionate-, butyrate- and acetate-degradation as well as on methane yields were studied.

4.1 Effects of $p\text{CO}_2$ on glucose-fermenting bacteria (II)

The fermentation of glucose to volatile fatty acids, CH_4 and CO_2 was studied in batch cultures at six different levels of

$p\text{CO}_2$ in the interval 0 - 1 atm. The rate of glucose degradation was 550 mg/l·h in N_2 and 415 mg/l·h in CO_2 , i.e. a difference of 30 %. However, while glucose degradation was completed after 6 - 8 h the further decomposition of acids to $\text{CH}_4 + \text{CO}_2$ required 4 - 10 days. Therefore, glucose degradation is clearly not rate-limiting, irrespective of $p\text{CO}_2$. This is in accordance with the literature results and expected on thermodynamical grounds, as discussed in the literature review (p 30).

The proportions of produced acids (HAc, HPr, HBut) from glucose were not much affected by $p\text{CO}_2$. This indicates that the capacity of methanogens to keep a low $p\text{H}_2$, i.e. produce methane from $\text{H}_2 + \text{CO}_2$, was not inhibited by $p\text{CO}_2$. Otherwise increased amounts of reduced acids would be expected at high $p\text{CO}_2$ in accordance with the theories on interspecies hydrogen transfer.

4.2 Effects of $p\text{CO}_2$ on propionate- and butyrate-oxidizing acetogens (II)

The rates of propionate- and butyrate-degradation at $p\text{CO}_2 = 0 - 1$ atm were studied in batch cultures, either as a continuation of glucose-fermentations or as fermentations starting with mixed acid substrates. Degradation rates are summarized in Fig. 2.

As discussed in the literature review (p 10) the oxidation of propionic and butyric acid to acetic acid are thermodynamically very unfavourable and low rates should be expected. Little information on the degradation of these acids is available. Kugelman and Chin (1971, data from McCarty) found degradation rates of 150 - 300 mg HPr/l·d and 330 mg HBut/l·d following spike injection of acids to continuous sewage sludge fermentations. Similarly, Kaspar and Wuhrman (1978) reported 50 mg/l·d in sewage sludge and 340 mg/l·d after propionate addition. These experiments were carried out without gas phase control, probably $p\text{CO}_2 = 0.4 - 0.5$ atm, and should therefore be comparable

to our values of around 170 mg/l·d each of HPr and HBut.

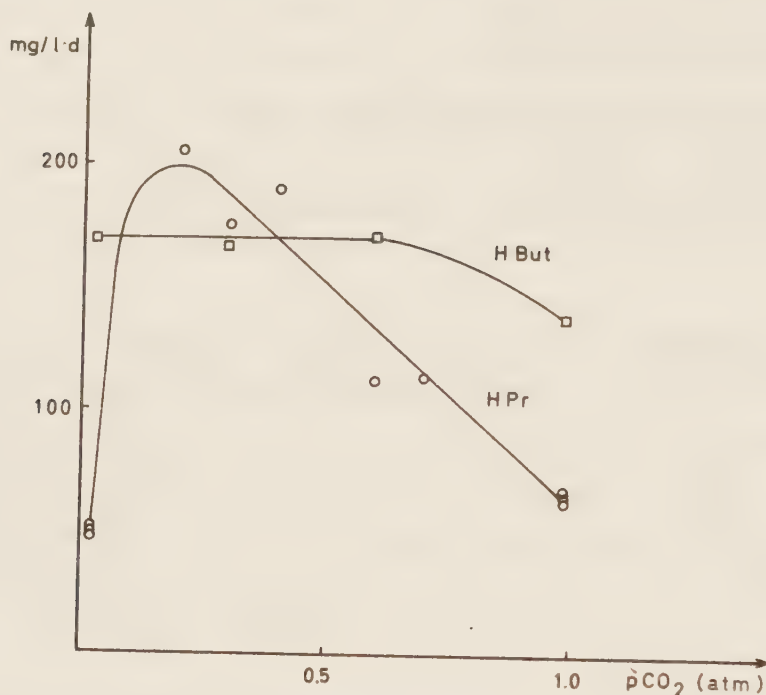


Fig 2. Rates of degradation of propionic and butyric acid as a function of $p\text{CO}_2$ in batch cultures

The rate of propionate degradation depended heavily on $p\text{CO}_2$. Inhibition at high $p\text{CO}_2$ should be expected from eqn (4) but, unexpectedly, degradation rates also decreased significantly at very low levels of $p\text{CO}_2$ with optimum rates around 0.2 atm CO_2 . The reason for this may be that some CO_2 -dependent reaction takes place during propionate-degradation and at the same time, methanogens compete for the CO_2 produced according to eqn (4).

Butyrate-degradation was not much affected by $p\text{CO}_2$; the degradation was slightly slower at 1 atm CO_2 . This is in agreement with eqn (5), which shows that CO_2 is not formed during butyrate degradation.

4.3 Effects of $p\text{CO}_2$ on acetate degradation by methanogens (III)

Methane production from acetate was studied in batch as well as continuous cultures. One example of a set of batch fermentations at three levels of $p\text{CO}_2$ is shown in Fig. 3. Degradation rates from all batch fermentations are shown in Fig. 4.

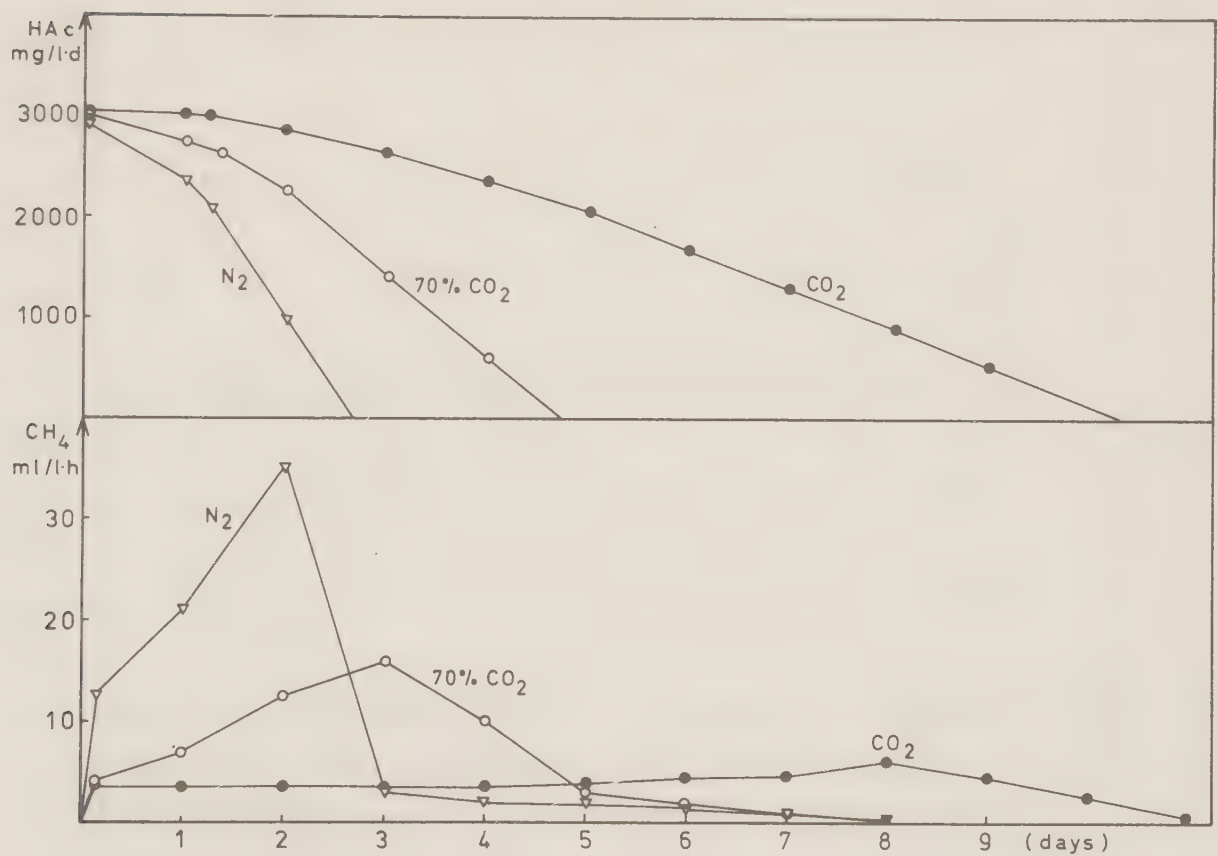


Fig 3. Rates of acetate degradation and methane production in batch fermentors sparged with N_2 , 70% CO_2 /30% N_2 and CO_2 , respectively

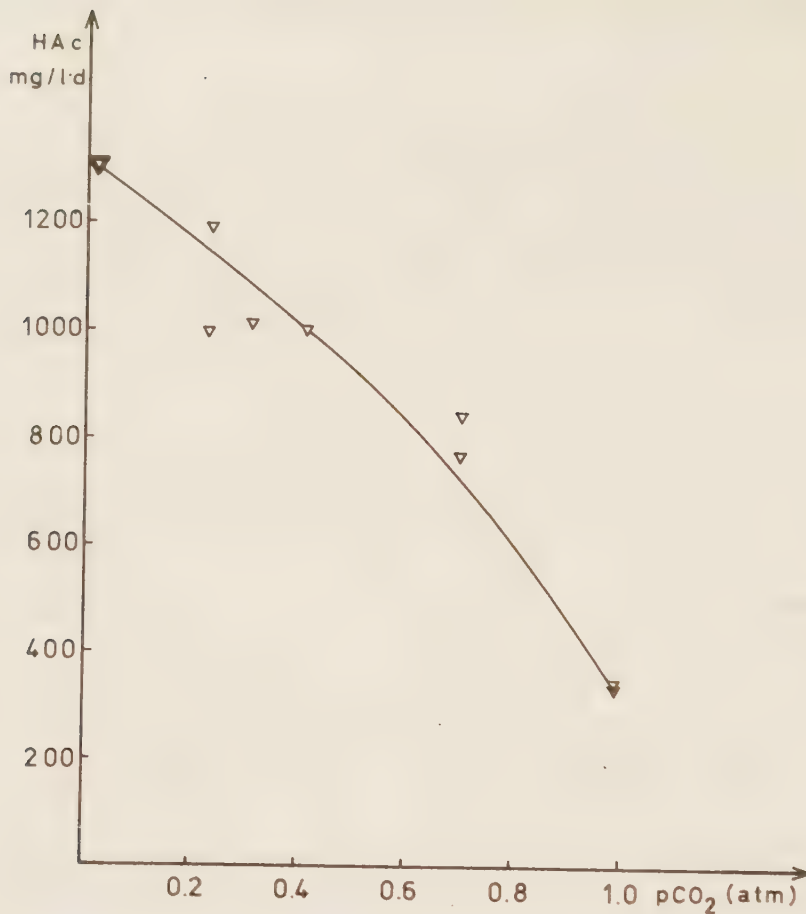


Fig 4. Rates of acetate degradation as a function of pCO₂

In Table 1 some of the values of acetate degradation rates in this work are compared with some literature data. Obviously, the data are heavily dependent on the type of microbial culture, cultural conditions, media and gas phase composition. In some cases the gas phase was not stated and assumed to be around 50 % CO₂/50 % CH₄. In other cases only the initial gas phase was known, and this probably changed due to gas evolution. Therefore, previous data were not usable for evaluating the possible influence of CO₂ on acetate degradation. Results from this work suggest a significant inhibiting effect of pCO₂ with a four-fold difference in the acetate degradation rate at 1 atm CO₂ compared to low values of pCO₂.

Table 1. Rates of mesophilic acetate degradation

Rate mg HAC/l·d	Cultural condition	Gas phase	Reference
112	pure culture (Methanosarcina)	N ₂	Weimer and Zeikus (1979)
90	pure culture ("Fat rod")	pCO ₂ = 0.2 atm	Zehnder et al (1980)
300-600	acetate enrichment	pCO ₂ ~ 0.4 atm	van den Berg et al (1976)
480	- " -	N ₂	Baresi et al (1978)
3800	- " -	pCO ₂ = 0.2 atm	Finney and Evans (1975)
280	spike injection of acetate in sewage sludge digester	pCO ₂ ~ 0.5 atm ^A	McCarty, as quoted by Kugelman and Chin (1971)
600	- " -	pCO ₂ ~ 0.3 atm	Kaspar and Wuhrmann (1978)
960-3400	- " -	pCO ₂ ~ 0.5 atm ^A	Stafford et al (1980)
4000	spike injection of acetate in cellulose enrichment	pCO ₂ ~ 0.5 atm ^A	Winter and Cooney (1980)
2000-2500	acetate enrichment, con- tinuous	N ₂ (sparging)	This work
1000-1500	glucose enrichment, batch	N ₂ (sparging)	- " -
350	- " -	CO ₂ (sparging)	- " -

^A estimated value

4.4 Effects of $p\text{CO}_2$ on methane yields (II, III)

$p\text{CO}_2$ was found to affect the rate of substrate degradation (mainly HAc and HPr) as well as the rate of methane production, as shown for acetate degradation in Fig. 3. Also, the total amounts of methane produced during each fermentation were measured and methane yields calculated as shown in Fig. 5.

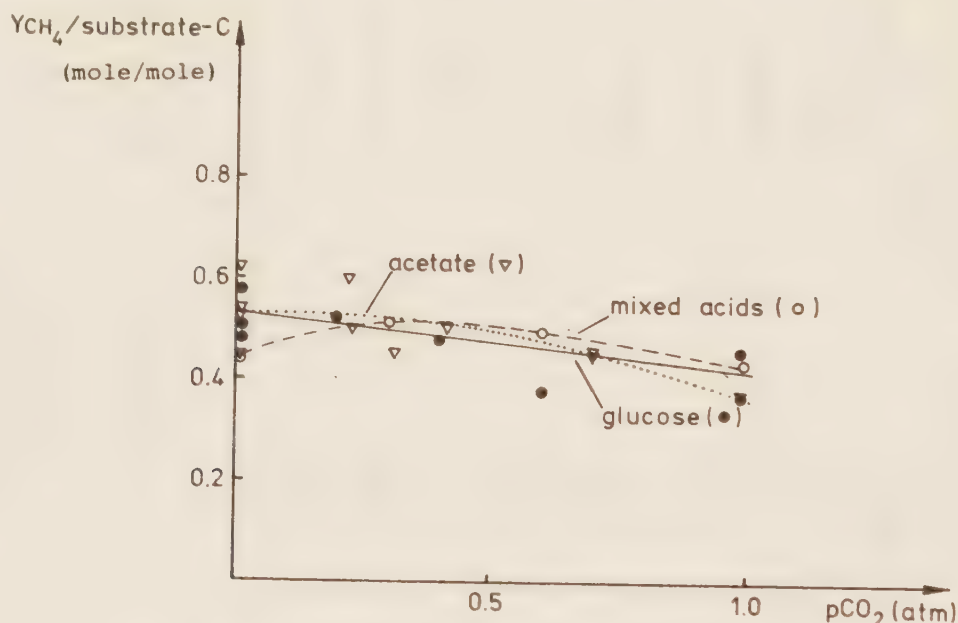


Fig 5. Effects of $p\text{CO}_2$ on methane yields from glucose and fatty acids. Yields are expressed as percentage of substrate-carbon found in CH_4

Theoretically, one mole of substrate-carbon should form 0.5 mole of both CH_4 and CO_2 . In fermentations at low $p\text{CO}_2$ yields were around the theoretical value providing negligible amounts of methane were formed from cell components or yeast extract. At high levels of $p\text{CO}_2$ yields were 20 - 30 % lower. One exception to this pattern was found in methane production from mixed acids. In this case the methane yield was somewhat lower during N_2 -sparging, probably related to the poor degradation of propionate in N_2 as discussed above.

5. Effects of $p\text{CO}_2$ in thermophilic methane fermentations (IV)

The rates of acetate utilization by methanogens were studied in combinations of $p\text{CO}_2 = 0 - 1$ atm and temperatures between 55 and 70° C. Generally, the same pattern of CO_2 -effects were found at 55 and 60° as in mesophilic fermentations. Degradation rates were 3 - 4 times greater in N_2 -atmosphere compared to CO_2 -atmosphere although thermophilic rates at all $p\text{CO}_2$ -values were about 50 % greater than the corresponding mesophilic rates. At temperatures between 65 and 70° C $p\text{CO}_2$ had a strong effect. At high $p\text{CO}_2$ (0.7 and 1.0 atm) the degradation rates of acetate were very low (0 - 150 mg/l·d) while rates were high (670 - 1800 mg/l·d) at low $p\text{CO}_2$ (0 and 0.3 atm). The implication of these results as well as further adaption to extreme temperatures will be discussed below.

An extended study was made at 55° C and $p\text{CO}_2 = 0.3$ atm to measure degradation rates of acetic, propionic and butyric acid as well as methane yields. Degradation rates were dependent on the type of culture used and will be discussed below in connection with the adaption of thermophilic cultures. Methane yields were found to be lower during thermophilic compared to mesophilic fermentations. While yields were about 0.5 mole CH_4 /mole substrate-carbon at 35° C, $p\text{CO}_2 = 0.3$ atm they were 0.30 - 0.35 mole/mole at 55° C, $p\text{CO}_2 = 0.3$ atm.

6. Mechanism for CO_2 -effects (I - IV)

The mechanism(s) by which CO_2 affect bacteria involved in methane fermentations are not known but certain speculations can be made, based on CO_2 -effects on other microorganisms and experimental data in this work.

Inhibition of methanogens at high $p\text{CO}_2$ is likely to be a direct effect of CO_2 due to the immediate response in methane production and acetate utilization to changes in $p\text{CO}_2$ (I).

Accumulation of toxic compounds as a result of CO_2 -sparging would effect the methane production gradually.

One possible explanation is that CO_2 affects the redox potential (E_h) and it is known that methane-producing cultures require very low E_h . E_h was measured continuously in all batch fermentations and was usually -200 to -250 mV, corresponding to electrode potentials, E_c , between -440 and -490 mV. According to the literature (see the literature review) methane production works well at E_c between -450 and -550 mV with an optimum between -510 and -530 mV. However, in this work, no clear connection between E_h and pCO_2 , acid degradation rates or methane production was found (II,III).

As discussed by Enfors and Molin (1981), CO_2 may affect bacteria in two different ways: by inhibiting intracellular, critical enzymatic reactions or by affecting the permeability of cell membranes. According to the latter theory, CO_2 would dissolve in cell membranes and impair membrane function by increasing its fluidity (Enfors and Molin 1978). Since high temperatures are also believed to increase membrane fluidity it would be expected that fermentation at reduced pCO_2 should increase the temperature maximum. Such results were demonstrated in methane production from acetate at 55 - 70° C (IV) and this should support the theory that CO_2 affects cell membranes of methanogens. Since it is known that high hydrostatic pressures decrease membrane fluidity a CO_2 -inhibited culture may be reverted back to normal fermentation rates by applying a high hydrostatic pressure on the culture, as demonstrated by Enfors and Molin (1978). This technique was tested on a methane-producing culture by applying 150 - 200 atm He on a CO_2 -inhibited culture (unpublished data). However, no positive reversal of CO_2 -inhibition was demonstrated, but due to technical problems to control fermentations, e.g. control of pH and pCO_2 , at very high pressures, results were uncertain.

7. Effects of $p\text{CH}_4$ in mesophilic methane fermentations (I, III)

In the initial studies (I) discussed above methane production from glucose in continuous culture at 0.85 - 0.90 atm CH_4 was studied. No inhibition of methane production or any acid accumulation was found as a result of high $p\text{CH}_4$.

Another test was made by sparging CH_4 in a batch fermentation degrading acetate to methane (III). The acetate degradation rate was compared to a standard N_2 -sparged fermentation. The rate at 1 atm CH_4 was found to be around 80 % of the rate in N_2 . Consequently, CH_4 was considerably less inhibiting on acetate degradation than CO_2 ; 1 atm CH_4 was comparable to 0.3 atm CO_2 .

8. Practical approaches to obtain low $p\text{CO}_2$

Most applications of methane fermentations are carried out at atmospheric pressure with 30 - 50 % CO_2 in the gas phase, i.e. $p\text{CO}_2 = 0.3 - 0.5$ atm. However, in the lower parts of large fermentors $p\text{CO}_2$ may be considerably higher due to the hydrostatic pressure and, if a gas dome is used, $p\text{CO}_2$ may increase further. Thus, $p\text{CO}_2$ around 1 atm is likely in many applications.

If it is desirable to reduce $p\text{CO}_2$ to obtain the faster rates and higher methane yields demonstrated in this work, one way is to use the gas recirculation system suggested in (I). If the gas is scrubbed to remove CO_2 , recirculated to the fermentor and contact established between gas and liquid by mixing, fermentation could be carried out at around $p\text{CO}_2 = 0.2$, $p\text{CH}_4 = 0.8$ atm which should give optimum rates and yields. Two drawbacks are inevitable: one is the poor buffering capacity due to bicarbonate removal; the other the energy requirement for mixing and CO_2 -scrubbing. If substrates containing large particles and flocs are degraded it is questionable if any imposed gas phase will reach the inner parts of the particles. An alternative to

a gas recirculation reactor is fermentation at reduced pressures, as suggested by Finney and Evans (1975). Pressures around 0.4 atm should be optimal, although the practical difficulties in large fermentors are obvious.

THERMOPHILIC METHANE FERMENTATION (IV)

1. Introduction

Some general aspects on thermophilic methane fermentation, its advantages and drawbacks, were summarized in the literature review (p 21).

In this work three aspects of thermophilic methane production were studied: the adaption of cultures to thermophilic conditions, the effects of $p\text{CO}_2$ on acetate degradation at $55 - 70^\circ\text{C}$ (as discussed in the preceeding section, p 49), and methane production at extreme temperatures, $70 - 85^\circ\text{C}$.

A common practice of obtaining thermophilic cultures is to start with mesophilic sewage sludge, manure, rumen fluid etc. The temperature may be increased from mesophilic to thermophilic levels in a slow and step-wise manner. For example, Pfeffer (1974) developed a culture degrading domestic refuse at 60°C by increasing the temperature $1 - 1.5^\circ\text{C}$ each week. Similarly, Cooney and Wise (1975) increased the temperature by 3°C every 3 weeks until reaching 65°C . In these examples thermophilic cultures were developed that degraded waste faster and with higher gas yields than mesophilic cultures. It was suggested that results obtained by others, e.g. Maly and Fadrus (1971), where thermophilic cultures were not much better than mesophilic ones, may be due to insufficient adaption of cultures. However, Varel et al (1977) rapidly developed cultures degrading cattle waste at 55°C . Starting with mesophilic sewage sludge, cattle waste and rumen fluid, the temperature was immediately increased to 55°C and rapid methane production was obtained after 6 days.

The microbiology of thermophilic methanogenesis is poorly understood. It is often stated, e.g. by Stafford et al (1980) that one set of bacteria is active at mesophilic temperatures and another set at thermophilic temperatures and neither group

is favoured at temperatures in between. Only two thermophilic methanogens are presently known in pure culture. One is Methanobacterium thermoautotrophicum, an autotrophic, extreme thermophile growing on $H_2 + CO_2$ with a temperature optimum at 65 - 70° C (Zeikus and Wolfe 1972). The other is a strain of Methanosarcina utilizing acetate, methanol and methylamines but not $H_2 + CO_2$ and a temperature optimum at 50° C (Zinder and Mah 1979).

Most data concerning thermophilic methane production from organic substrates, as reviewed by Buhr and Andrews (1977), suggest a temperature optimum around 60° C and a temperature maximum around 65° C. In a natural ecosystem, a hot-spring algal-bacterial mat, Ward (1978) found a temperature optimum at 45° C and a maximum around 65° C. However, autotrophic methane formation by Methanobacterium thermoautotrophicum may occur at higher temperatures and recently autotrophic methane production in hot springs at over 90° C has been reported (Zehnder 1981).

2. Adaption of cultures to thermophilic methane production

A study was made to determine how fast various mesophilic cultures may adapt to 55° C compared to a culture already working at this temperature. Four cultures were used:

- A thermophilic culture (55°) from a pilot scale digester treating sugar-beet wastes containing complex carbohydrates
- This culture, after storing at room temperature for 5 months
- A mesophilic glucose-enrichment, originally from sewage sludge, but cultivated continuously on glucose for 4 years
- Mesophilic digesting sewage sludge

In all cases the temperature was immediately increased to 55° C and semicontinuous cultivation on a glucose-based

substrate at pH 6.8 started.

The thermophilic culture, as expected, produced methane from glucose without lag-phase and without any acid accumulation. Storing of the culture at room temperature had no significant effect; there was a temporary accumulation of small amounts of acetate (500 mg/l) during the first 2 days, then glucose became completely degraded. Also the mesophilic glucose-enrichment and sewage sludge produced methane immediately after a temperature increase to 55° C. The lag periods before glucose was completely utilized were longer, 5 and 14 days respectively, with temporary accumulation of acids.

Besides methane production from glucose, the degradation of volatile fatty acids to methane by acetogenic and methanogenic bacteria were studied. The degradation rates of acetate, propionate and butyrate were compared in three cultures: mesophilic glucose-enrichment (35° C), this enrichment after 1 week at 55° C and the thermophilic sugar-beet waste culture.

The glucose-enrichment adapted to 55° C was found to degrade acetate and butyrate at the same rate as the original culture while propionate was not degraded at all in the adapted culture. The waste-culture degraded acetate and butyrate 2 - 3 times faster than the other cultures which may be due to higher specific activity and/or higher cell density. However, as pointed out above (p 49), methane yields were lower in thermophilic than mesophilic cultures.

These results suggest that mesophilic cultures may easily and rapidly adapt to thermophilic conditions without a stepwise increase in temperature. It is unlikely that there are considerable numbers of thermophiles in mesophilic sewage sludge, although thermophiles may enter with the raw sludge. However, after 4 years of continuous cultivation in the laboratory their numbers should be insignificant and require several weeks to reach numbers sufficient for any substantial methane

production. The rapid adaption demonstrated in this work therefore suggests that the mesophilic bacteria involved have the capacity to switch to thermophilic conditions with short lag periods.

3. Methane production at extreme temperatures

As discussed above (p 49), methane production from acetate at 65 - 70° C worked at low $p\text{CO}_2$ but not at high $p\text{CO}_2$ possibly indicating CO_2 -effects on cell membranes. Therefore, a study was made to determine the temperature maximum for methane production from glucose in a semicontinuous fermentation with N_2 -sparging.

The temperature was increased stepwise from 70° C to 85° C. The methane fermentation worked, also at 80° C, although acetate accumulated. At 85° C glucose degradation stopped but traces of methane were still produced. Methane yields decreased with increasing temperature. While yields were around 100 % (= 0.5 mole CH_4 per mole substrate-carbon) at mesophilic temperatures, yields at 55° C were 50 - 70 % and 20 - 30 % at 80° C. The experimental data suggest that methane was partly produced from acetate at 70-74° C and mainly from acetate at 76-80° C rather than from $\text{H}_2 + \text{CO}_2$.

These results are quite different compared to literature data. The temperature maximum for methane production from complex materials is usually found around 65° C although autotrophic methane production from $\text{H}_2 + \text{CO}_2$ may be found in habitats such as hot springs at considerably higher temperatures.

METHANE PRODUCTION FROM GREEN MACRO-ALGAE (V)

1. Background

Biomass, e.g. agricultural and forest residues, municipal wastes and energy crops is a likely future source of significant amounts of fuels and industrial chemicals. Several methods of converting biomass are possible, anaerobic digestion to produce methane being one. Some of the advantages of using methane production from biomass are:

- a wide range of biomasses with varying dry matter content are suitable for methane fermentation
- as complex, mixed cultures, methane producing cultures have the capacity to simultaneously degrade a wide variety of complex organic compounds present in most biomasses
- the products, $\text{CH}_4 + \text{CO}_2$, are spontaneously separated from the fermentation broth thus avoiding separation problems such as extraction and distillation and permitting high substrate concentrations
- methane fermentations are carried out during non-sterile conditions
- inexpensive reactors and energy-conserving fermentation methods, resulting in high net energy yields, may be used

Much research and development has been carried out and is going on regarding methane production from energy crops. Some interesting examples, representing land-based and water-based crops, are briefly summarized here:

Work in New Zealand (Stewart 1979, Badger et al 1979) suggests that the value of fuel produced from energy crops in this country would be greater than alternative production of

food. The cost of producing biogas from crops may be lower than producing it from waste materials, even though no value is put on the waste. The main reason is that energy crops may be chosen for their suitability for digestion while many waste materials give poorer yields and slower degradation. Furthermore, energy crops may be grown close to the digester to minimize handling costs and the digested slurry is returned as fertilizer. A variety of crops have been tested in laboratory and pilot plant experiments e.g. oats, maize, hay, sugar-beet leaves and lucerne. Based on crop yields at 18 - 20 ton/ha.yr and biogas yields of 450 l/kg (70 % CH_4 , 15 days retention time) it was found that biogas and compressed methane produced from crops is economically competitive with fossil fuels. Net energy yields of 70 % are reported; including crop production, fermentation, gas scrubbing and compression (Stewart et al 1981).

As suitable land in most countries may not be available for energy crop production an alternative may be to produce crops at sea. It is believed (Ryther et al 1977 and others) that very high crop yields - around 50 tons/ha.yr are possible at sea - although a new technology has to be developed. In a large project in the USA, the "Energy from Marine Biomass Program", the technical and economic feasibility of methane production from kelp biomass is being studied (Flowers and Bryce 1977, Wise et al 1976). The giant brown kelp, Macrocystis pyrifera, will be cultivated in the open ocean on artificial structures. Nutrients will be provided by natural or artificial upwelling of deep ocean water and the kelp periodically harvested by special ships. It is estimated that harvests of around 850 tons wet weight/ha.yr could be achieved. This would correspond to 60 ton VS/ha.yr.

It should be emphasized that although high crop yields may be obtainable the large scale production of methane - or any other fuel - from energy crops requires very large areas. For example, a 50,000 ha mariculture system producing giant kelp as above, would have produced only 0.04 % of the U.S. energy demand in

1970 (Wise et al 1976).

A new approach to the cultivation of seaweed in Scandinavian waters was suggested by von Wachenfeldt (1980). The intention is to cultivate seaweeds such as the green algae Ulva, Chaeotomorpha and Cladophora in a free floating condition in sheltered coastal waters thus avoiding the expensive structures and other problems of open ocean farming. By utilizing a succession of fast-growing algae, each adapted to maximum growth during a certain period of the total growth season, several harvests during an 8 - 9 month period each year should be possible. Small scale cultivation experiments suggest yields of around 50 ton VS/ha.yr.

2. Anaerobic digestion of green macro-algae

The purpose of this work was to demonstrate the feasibility of producing methane from the green algae Ulva, Chaetomorpha and Cladophora and to determine basic fermentation parameters such as yields, retention times and loading rates in conventional semicontinuous, completely mixed, mesophilic and thermophilic fermentations.

2.1 Inocula and start-up procedure

A mixed inoculum was prepared, composed of digesting sewage sludge, rumen contents, salt and brackish water sediments and rotting seaweeds. The substrate consisted of ground, mixed green algae without any nutrient additions. The fermentors were initially sparged with N_2 and filled with inoculum and only a small volume substrate. After 10 days daily substrate additions at low loading rates were started. With this method mesophilic as well as thermophilic methane production started immediately and no accumulation of volatile fatty acids occurred.

2.2 Retention times and loading rates

Retention times ranging from RT = 27 days to RT = 11 days were tested during a 12 month period. The corresponding loading rates were from 1.1 to 2.6 g VS/l·d. Both the mesophilic and the thermophilic fermentations worked at all tested retention times and loading rates with a possible slight decrease in gas yield at RT = 11 days. Therefore, retention times of 12 - 15 days should be suitable in methane production from green algae in high-rate digestors.

2.3 Gas yields and VS-reduction in mesophilic fermentation

In mesophilic fermentation the methane yield was 250 - 350 ml CH₄/g VS_{added} and the gas composition approximately 60 % CH₄, 40 % CO₂. The VS-reduction was around 50 - 55 %. These data were found irrespective of loading rate and retention time. The methane yields may also be expressed as 450 - 700 ml CH₄/g VS_{degraded} and the volumetric methane production 300 - 370 ml CH₄/l·d at 11 days RT. In Table 2 yield data from this work are compared with corresponding literature data regarding methane production from brown and red algae and some land-based energy crops.

When producing methane from energy crops yields are obviously very important - harvest yields when growing the crop as well as methane yield in anaerobic digestion. Equally important is to minimize the energy input in cultivation, handling and processing the crop so as to obtain a reasonable net energy yield.

As for methane production from seaweeds yields around 300 ml CH₄/g VS_{added} and 50 % VS-degradation seem to be the best results presently reported. Thus, there is a potential to increase yields considerably and at the same time reduce the problem to handle and take care of fermentation residues - which have very high salt contents. A main research need is

Table 2. Yields of methane in anaerobic digestion
of seaweeds and other energy crops

Biomass	RT days	Loading rate g VS/l·d	Temp °C	Yield ml CH ₄ / g VS _{added}	VS-re- duction %	Reference
<u>Ulva + Cladophora</u> <u>+ Chaetomorpha</u>	12-15	2.0-2.5	35	250-350	50-55	This work
<u>Macrocystis pyrifera</u>	18	1.6	35	275-310	48-57	Klass et al (1979)
<u>Laminaria saccharina</u>	25	1.1-1.6	37	205-220	49-53	Troiano et al (1976)
<u>Gracilaria tikvahiae</u>	30-60	0.54	30 ± 5	130-200	26-48	Hanisak (1981)
Hay	18	batch	37	295	70	Badger et al (1979)
Sugar-beet leaves	21	batch	37	297	79	- "
Lucerne	17	batch	37	247	69	- "

therefore to find inexpensive ways to increase methane yields. Among the possible approaches is to operate digestors at high cell densities, to pretreat the substrate to increase digestibility and to find better inocula and optimum environmental conditions for methane production from seaweeds.

2.4 Thermophilic fermentation

In thermophilic fermentation the VS-reduction was also around 50 % but the methane yield was somewhat lower than in the mesophilic culture. The thermophilic culture was also more sensitive to disturbances and concentrations of VFA were generally higher. Therefore, no advantages were found with thermophilic methane production from algae, compared to mesophilic fermentation.

CONCLUSIONS

- The effects of $p\text{CO}_2$ and $p\text{CH}_4$ in the interval 0-1 atm on methane fermentation kinetics and yields were studied. A method based on measurement of substrate utilization rates in batch fermentations was developed.
- The rates of degradation of glucose, acetate, propionate and butyrate in methane fermentations were found to be 10,000 - 13,000; 350 - 1,300; 50 - 200 and 140 - 170 mg/ $\text{l}\cdot\text{d}$, respectively, with variations due to $p\text{CO}_2$. The great differences in rates of degradation of these substrates are consistent with thermodynamics: $\Delta G^{\circ'}_{\text{glucose}} = -60 \text{ kcal/mole}$, $\Delta G^{\circ'}_{\text{HAc}} = -7.4 \text{ kcal/mole}$, $\Delta G^{\circ'}_{\text{HPr}} = +18.5 \text{ kcal/mole}$ and $\Delta G^{\circ'}_{\text{HBut}} = +11.5 \text{ kcal/mole}$.
- Propionate- and butyrate oxidation were rate-limiting steps; in particular the degradation of propionate at low or high $p\text{CO}_2$ was very slow. Methane production from acetate was 3 - 10 times faster. However, this does not necessarily mean that the proton-reducing, acetogenic steps are the crucial reactions. Acetate is still the major precursor of methane while propionate and butyrate normally are minor intermediates.
- CO_2 was found to inhibit acetate degradation, with a four-fold difference in rates at low $p\text{CO}_2$ compared to 1 atm CO_2 . Similarly, CO_2 inhibited propionate degradation but in this case degradation rates were low at high as well as at very low $p\text{CO}_2$ (N_2 -atmosphere).
- $p\text{CO}_2$ affected methane yields on glucose and fatty acid substrates with 20 - 30 % lower yields in 1 atm CO_2 compared to low $p\text{CO}_2$.
- The same degree of CO_2 -inhibition was found in thermophilic methane production ($55\text{-}60^\circ\text{C}$) as in mesophilic fermentations (35°C).

- These results suggest that $p\text{CO}_2$ should be regarded as an important cultivation parameter and in order to obtain optimum fermentation rates and methane yields, fermentations should be carried out at around 0.2 atm CO_2 .
- CH_4 was not significantly inhibitory compared to CO_2 . Therefore, to avoid CO_2 -inhibition fermentations may be carried out in a CH_4 -atmosphere. This was achieved by using a gas-recirculation system in which CO_2 was absorbed in alkali and CH_4 returned to the reactor.
- The adaption of mesophilic cultures to thermophilic conditions was found to proceed rapidly as the result of an immediate temperature increase from 35°C to 55°C . The results suggest that some mesophilic bacteria have the capacity to "switch" to thermophilic conditions rather than there is a special thermophilic flora active only at high temperatures.
- The temperature maximum for methane production from acetate was around 65°C at $p\text{CO}_2 = 0.7\text{--}1.0$ atm. At $p\text{CO}_2 = 0\text{--}0.3$ atm the temperature maximum was over 70°C . These results suggest that CO_2 is inhibitory by a mechanism involving cell membranes.
- Methane production from glucose was possible at temperatures up to 85°C in N_2 -atmosphere. Since literature data state a temperature maximum around 65°C these results suggest a very strong effect of $p\text{CO}_2$ at high temperature and/or the occurrence of unique bacteria in the cultures. The inoculum originated from a sugar-beet waste plant where sugar-beets are treated at temperatures around 70°C .
- Methane production from marine, green algae at 35°C and 55°C was studied in semi-continuous fermentations. By using a wide range of inocula such as sediments, rumen contents and sewage sludge a rapid start-up was possible.

As substrate ground algae without any nutrient additions were used.

- Based on one year of continuous, mesophilic methane production in conventional digestors, the following conclusions were made: Suitable retention time 12-15 days, gas yield 250-350 ml CH₄/g VS_{added} and VS-reduction around 50-55 %. These data are comparable or somewhat better than literature data regarding methane production from brown and red algae.
- No advantages were found with thermophilic methane production over mesophilic fermentation. In methane production from algae the rates and yields were the same or somewhat lower in thermophilic fermentations. In methane production from fatty acids the rates were higher but the methane yields were considerably lower in thermophilic compared to mesophilic fermentations.

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EFFECTS OF CARBON DIOXIDE AND METHANE ON METHANOGENESIS

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Effects of Carbon Dioxide and Methane on Methanogenesis

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Summary. A mixed microbial culture, enriched from sewage sludge, was cultivated on a glucose medium in a high turbulence fermentor at constant temperature, pH and loading rate. Effects of high $p\text{CO}_2$ (0.95 atm) and $p\text{CH}_4$ (0.90 atm) were compared to those of low $p\text{CO}_2$ (0.05 atm) and $p\text{CH}_4$ (0.05 atm). Generally, rapid increases in $p\text{CO}_2$ resulted in rapid decreases in methane production and, conversely rapid decreases in $p\text{CO}_2$ by N_2 -sparging resulted in rapid increases in methane production. Decreased values of methane production were also accompanied by acetic acid accumulation. No inhibiting effects of high $p\text{CH}_4$ were detected. A method to obtain low $p\text{CO}_2$ by gas recycling combined with CO_2 -absorption is proposed.

Introduction

Methane fermentations of organic matter are carried out by different groups of anaerobic bacteria. Fermentative bacteria hydrolyze complex organic compounds and the products are fermented mainly to acetic acid, hydrogen and carbon dioxide. More reduced compounds like propionic acid, butyric acid, ethanol and lactic acid may be formed as well, but these compounds can be oxidized to acetic acid by so called acetogenic bacteria of which only two are known, oxidizing ethanol and butyric acid, respectively (M.P. Bryant, personal communication). Methanogenic bacteria form methane mainly from hydrogen, carbon dioxide and acetic acid. Acetate is regarded as the major precursor of methane and accounts for 70–75% of its production in sewage sludge and sediments (Smith and Mah, 1966; Jeris and McCarty, 1965).

The methanogenic step is generally regarded as rate-limiting in methane fermentations, at least of soluble wastes (Lawrence and McCarty, 1969). The condition of the methanogenic bacteria is also crucial for fermentative and acetogenic activity according to recent theories on hydrogen transfer (Wolin, 1975; Zeikus, 1977; McInerney and Bryant, 1978). By consuming H_2 , thus keeping a very low hydrogen partial pressure, the methanogenic bacteria divert the fermentative activity towards the production of acetic acid. Furthermore, the low $p\text{H}_2$ is necessary for the acetogenic bacteria. In

digestion processes the generation time (g) for methanogenic bacteria has been estimated to be 2-11 days leading to minimum solids retention times of 3-16 days (Ghosh and Pohland, 1974). Also when an enriched methanogenic culture is grown on acetic acid media the growth is slow with minimal doubling times of 1.4-2.9 days (Lawrence and McCarty, 1969). Under certain conditions, however, methanogenic bacteria seem to have the capacity to grow much faster. For example, *Methanobacterium thermoautotrophicum* has been cultured on hydrogen and carbon dioxide with a "g" of 3 h (Zeikus and Wolfe, 1972) and *Methanobacterium* Strain AZ was cultured on hydrogen and carbon dioxide with a "g" of 5 h (Zehnder and Wuhrman, 1977). Also, in mixed methanogenic cultures enriched from sewage sludge and growing on hydrogen and carbon dioxide, fast growth (g = 6.8 h) has been achieved (Wise et al., 1978). These differences could, at least partly, be explained by the greater energy yields when methanogens grow on hydrogen and carbon dioxide ($\Delta G = -32.7$ kcal/mol) compared to growth on acetic acid ($\Delta G = -8.6$ kcal/mol) (Zeikus, 1977). However, fast growth (g = 6.7 h) has also been obtained on acetic acid media with mixed methanogenic cultures enriched from sludge (Finney and Evans, 1975). These results were achieved in a low-pressure fermentor and it was suggested that the transfer of carbon dioxide through bubble membranes is rate-limiting in anaerobic digestion. Fast growth and high loading rates were also possible on glucose media (Finney et al., 1976). Transfer of CO_2 across interfaces has also been suggested as an important parameter by Andrews and Graef (1971).

Thus, it has been suggested that carbon dioxide might have an inhibiting effect on methane fermentations. Inhibiting effects of methane have also been demonstrated (Brock and Zehnder, 1978). Any inhibiting effects of these gases should be very important since pCO_2 as well as pCH_4 could increase to several atmospheres in large digestors and even further in a gas dome. In this work the effects of high and low partial pressures of carbon dioxide and methane on methanogenesis and substrate consumption in a mixed fermentor-culture have been studied.

Material and Methods

Microbial Culture. A mixed microbial culture was originally obtained from a sewage sludge digester (Lund's Sewage Works). The culture was adapted to a glucose-based medium in a 13-liter stainless steel digester working with long retention times (30-60 days) and low loading rates (0.2-0.7 g glucose/liter day). The digester was operated semi-continuously; 10 ml substrate was fed with 1-2 h intervals. The temperature was held at 35°C and the culture was mixed by impellers at 200 rpm. The digester operated for about 12 months and was used as a source of inoculum for the fermentor cultures.

Growth Medium. In g/liter Glucose 16; yeast extract, 4.0; NH_4Cl , 2.0; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.3; KH_2PO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. Trace elements (mg/l): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.3; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.09; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.09; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.07; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.07. The substances were dissolved in distilled water. The medium was autoclaved at 120°C in three parts; glucose, phosphates, and the rest.

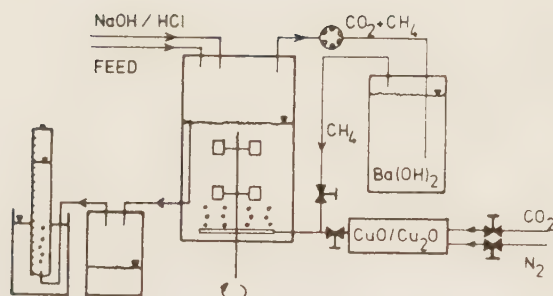


Fig. 1. Schematic diagram of fermentor equipped to obtain high $p\text{CH}_4$, $p\text{CO}_2$ or $p\text{N}_2$

Fermentor Cultivations. A glass stainless steel fermentor (Chemoform 4L), total volume, 7 l, effective volume, 2500 ml, was used. The temperature was constant at 37°C and the culture mixed by impellers at 800 rpm. Substrate was added in 10 ml increments with 1 h intervals. The retention time and loading rate was approximately 6 days and 3 g glucose/liter day, respectively. High partial pressures (0.95 atm) of CO_2 and low partial pressures (0.05 atm) of CO_2 and CH_4 were achieved by sparging CO_2 or N_2 through the fermentor at a rate of approximately 2 l/h. The gas was passed through a $\text{CuO}/\text{Cu}_2\text{O}$ -column at 360°C to remove oxygen traces. High partial pressures of CH_4 (0.90 atm) were achieved by recirculating fermentor gas with a peristaltic pump through a $\text{Ba}(\text{OH})_2$ solution to absorb CO_2 (Fig. 1). The resulting flow rate of CH_4 through the fermentor was approximately 2 l/h. The fermentor liquid level was kept constant by an overflow through which the product gases were also lead to an inverted, water-filled measuring cylinder.

Analytical Procedures. pH was measured in the fermentor with glass-calomel electrodes and controlled at 7.0 by automatic addition of 4 M NaOH or 4 M HCl. Gas analyses (N_2 , CH_4 , CO_2) were carried out by gas chromatography according to van Huyssteen (1967).

Fatty acids and alcohols were measured with a modified gas chromatographic method according to Carlsson (1973).

Glucose was measured by the hexokinase method (Schmidt, 1961).

Total cell mass (acid formers and methane formers) was determined by centrifuging at 30.000 g for 20 min after acidification to remove carbonate. The pellet was washed with NaCl (0.85 g/l), recentrifuged and dried overnight at 105°C .

Carbon contents of cells were analysed with a Carlo Erba elementary analyser. Sodium was analysed with a Varian-Techtron Atomic Absorption Spectrophotometer.

Results

1. Effects of High $p\text{CO}_2$. A mixed culture growing on glucose was exposed to a high $p\text{CO}_2$ achieved by carbon dioxide-sparging in three experiments: (1) 8 days of carbon dioxide-sparging (Fig. 2); (2) 3 days and 5 days of carbon dioxide-sparging both preceded and followed by nitrogen-sparging (Fig. 3); (3) 19 days of carbon dioxide-sparging (Fig. 4). Experiments (1) and (2) were started with fresh inoculum (100%) from the 13 l digester. A high $p\text{CH}_4$ -test [see below and experiment (3)] directly followed experiment (2) using the same culture.

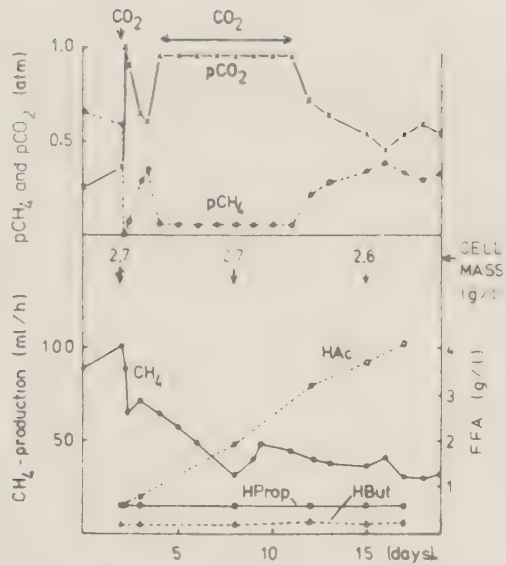


Fig. 2. Effects of high $p\text{CO}_2$ on methane and fatty acid production

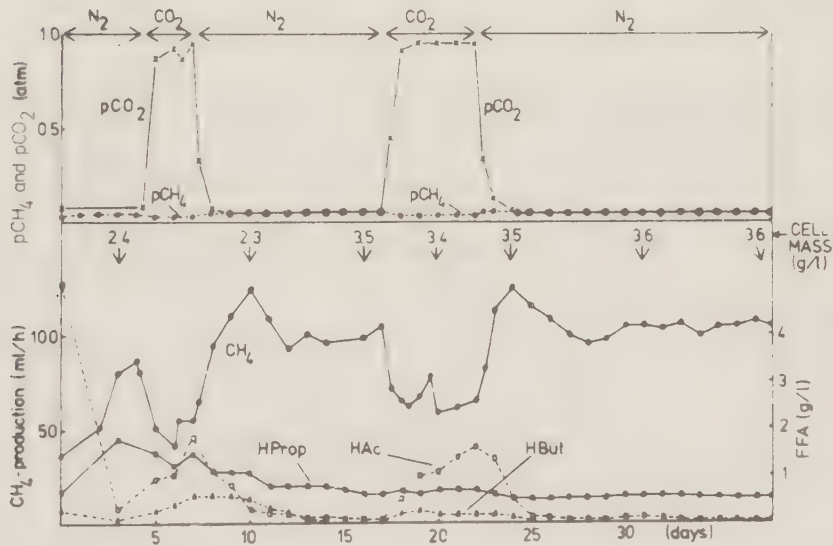
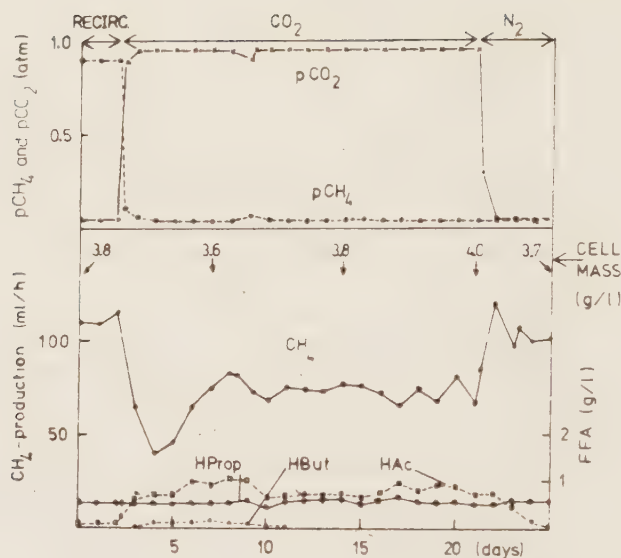


Fig. 3. Effects of alternating high $p\text{N}_2$ and high $p\text{CO}_2$ on methane and fatty acid production

In general, rapid increases in $p\text{CO}_2$ resulted in rapid decreases in methane production and, conversely rapid drops in $p\text{CO}_2$ by nitrogen-sparging led to rapid rises in methane production. Decreased methane production values were also accompanied by accumulation of acetic acid. In all cases, glucose was almost completely utilized (rest values around 40 mg/l) irrespective of gas atmosphere.

In the first test (Fig. 2) the fermentor was momentarily sparged with carbon dioxide to 100% in the gas phase. When $p\text{CO}_2$ had decreased to 0.6 atm after 2 days,

Fig. 4. Long-term effects of high $p\text{CO}_2$ on methane and fatty acid production



continuous CO_2 -sparging was started and continued for 8 days. Then the sparging was stopped and the $p\text{CO}_2$ allowed to decrease to approximately 0.5 atm. Methane production dipped sharply when carbon dioxide-sparging began and continuously decreased to about 30% of the original values. Some tendency to adapt to high $p\text{CO}_2$ occurred, but methane production did not increase when carbon dioxide-sparging was ceased. Acetic acid concentrations increased almost linearly to 4 g/l while propionic and butyric acid concentrations remained constant at 0.6 and 0.2 g/l, respectively. Cell mass remained constant approximately 2.7 g/l.

In the second experiment (Fig. 3) $p\text{CO}_2$ was changed more rapidly by alternating N_2 - and CO_2 -sparging. As in the first test, methane production dropped rapidly when CO_2 -sparging started. It increased again, though, when $p\text{CO}_2$ was rapidly lowered to 0.05 atm. The acetic acid concentration rose at approximately the same rate as in the first experiment during the high $p\text{CO}_2$ -periods. When the $p\text{CO}_2$ was lowered, acetic acid was consumed causing a temporary peak in methane production. Then the methane production stabilized at the same level as before the high $p\text{CO}_2$ -period. During N_2 -sparging acetic acid concentrations were very low, approximately 50 mg/l. Propionic acid concentrations stabilized at approximately 0.5 g/l, as in the first experiment, after initial higher values and was not, or only slightly, affected by $p\text{CO}_2$. Butyric acid concentrations increased clearly during periods of high $p\text{CO}_2$, remained very low (50 mg/l), however, during N_2 -sparging. Cell mass did not significantly change with changes in $p\text{CO}_2$. An estimation of how substrate-carbon was distributed in the different fermentation products under CO_2 - and N_2 -sparging is compiled in Table 1.

The effects of a 19-day period of high $p\text{CO}_2$ were examined in the third experiment (Fig. 4) which began, as the continuation of the recirculation-test reported below, with a high $p\text{CH}_4$. Rapid $p\text{CO}_2$ changes brought about the same effects as in the two earlier experiments plus an adaption to high $p\text{CO}_2$ after 2 days. The acetic acid accumulation, however, was different. As in the earlier cases, the acetic acid concentration

Table 1. Approximate distribution of substrate-carbon into products during high N_2 - and high CO_2 -conditions

Product	N_2 -sparging % of glucose-carbon	% of total carbon	CO_2 -sparging % of glucose-carbon	% of total carbon
CO_2	48	39	30	24
CH_4	48	39	30	24
HAc	0.3	0.2	20	16
HProp	4	3	4	3
HBut	0.1	0.1	3	2
Glucose	0.3	0.2	0.3	0.2
Cells	25	20	25	20
Total	126	101	112	89

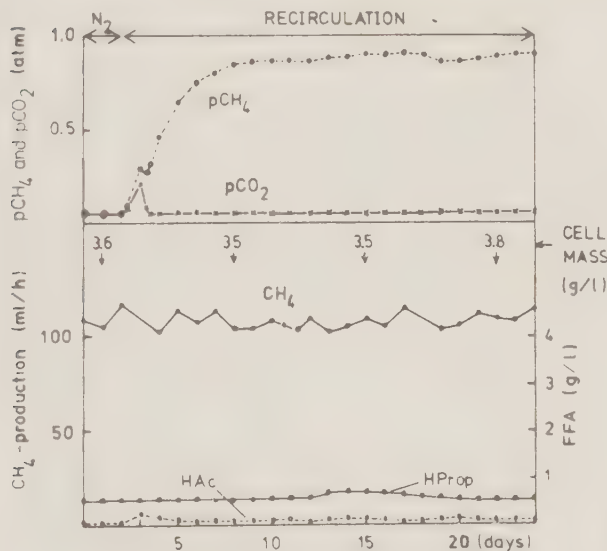


Fig. 5. Effects of high pCH_4 on methane and fatty acid production

increased immediately after increase in pCO_2 and at the same rate during the first day of high pCO_2 . Then, however, the accumulation rate decreased and acetic acid concentrations remained at approximately 800 mg/l during the whole high pCO_2 period. When the pCO_2 was lowered the result was the same as before; acetic acid concentrations decreased to the very low levels typical of N_2 -sparging. Also as before, propionic acid concentrations remained constant at approximately 0.5 g/l, independent of the pCO_2 . Butyric acid concentrations, however, were only slightly higher during the first week of the high pCO_2 and subsequently remained low at 30-90 mg/l despite the continued high pCO_2 . Cell mass first decreased by 5% and then increased by 10% during CO_2 -sparging.

2. *Effects of High pCH_4 .* After the two short-term periods of high pCO_2 and 16 days of high pN_2 , the gas recirculation system as described above was started and the effects

of high $p\text{CH}_4$ were studied (Fig. 5). $p\text{CH}_4$ gradually increased to 0.85-0.90 atm. The break in the $p\text{CH}_4$ -curve at 3 days was due to a temporary stop in the gas recirculation.

The methane production under high $p\text{CH}_4$, low $p\text{CO}_2$ conditions was approximately the same as under high $p\text{N}_2$, low $p\text{CO}_2$. Acetic acid concentrations were slightly higher; approximately 80 mg/l compared to 50 mg/l in an N_2 -atmosphere. The propionic acid concentrations stayed as unchanged at approximately 0.5 g/l in the methane atmosphere as they did in the carbon dioxide and nitrogen atmospheres and butyric acid concentrations were very low, 0-50 mg/l. The cell mass was also the same as it was in the nitrogen atmosphere.

Discussion

As shown by the sudden drop in methane production and the gradual increase in acetic acid concentration it can be concluded that the methanogenic bacteria were partly inhibited by the high $p\text{CO}_2$ (0.95 atm). The sugarfermenting bacteria were capable of completely utilizing glucose under high and low $p\text{CO}_2$ -conditions. According to several investigators (Wolin, 1975), a diversion of acid-production in favor of the production of more reduced compounds should be expected in mixed cultures where methanogenic bacteria are stressed until $p\text{H}_2$ increases. So was the case as butyric acid production increased during periods of high $p\text{CO}_2$. Propionic acid production, however, was not affected by high $p\text{CO}_2$, whereas, according to McInerney and Bryant (1978), in a disturbed system the concentration of propionic acid should increase before that of the butyric acid. Possibly, no acetogenic bacteria which would oxidize propionic acid were present in our case.

The average methane production under low $p\text{CO}_2$ conditions was 40, under high $p\text{CO}_2$, 27 ml/l/h a difference of 33%. Substrate-carbon was composed of glucose- (80%) and of yeast extract-carbon (20%). During nitrogen-sparging the substrate-carbon was completely utilized to give gas yield of 77% (100% gas yield is when all the substrate-carbon is converted to gas). During carbon dioxide-sparging only part of the yeast extract was expended; the gas yield was 49%, assuming equal production of methane and carbon dioxide during carbon dioxide-sparging and no assimilation of sparged gas.

Assuming that 70% of the produced methane was derived from the methyl group of acetic acid, 30% from hydrogen and carbon dioxide, the rate at which acetic acid was converted to methane in the nitrogen-atmosphere was 28 ml methane/l/h whereas 12 ml of methane/l/h were produced from hydrogen and carbon dioxide. In the CO_2 -atmosphere the substrate-glucose was completely expended, no hydrogen-production was detected by gas chromatography and the amount of hydrogen in the butyric acid was small. Therefore the methane production from hydrogen and carbon dioxide should be almost the same. That would mean 12 ml methane/l/h from hydrogen and carbon dioxide and 15 ml methane/l/h from acetic acid, or a 46% reduction in the utilization of acetic acid in the carbon dioxide-atmosphere as opposed to the N_2 -atmosphere. The differences in methane production and acetic acid utilization during high and low $p\text{CO}_2$ might be even greater than these figures indicate. Probably, the culture was substrate-limited in the low $p\text{CO}_2$ -experiments and therefore, the real

capacity for the culture to produce methane is not known. The kinetics of methane production as a function of gas phase composition is being continuously investigated.

An exception to the discussion on acetic acid accumulation above is the 19-day high $p\text{CO}_2$ -test. Acetic acid accumulated at the same rate as in earlier experiments only during the first day of high $p\text{CO}_2$. Then the accumulation rate decreased and acetic acid concentrations leveled off at around 800 mg/l, which corresponds to only 25% of the earlier acetic acid production. However, the decrease in methane production was approximately the same and accumulation of other compounds could not be detected. One explanation might be that acetic acid was produced in the same amounts as before but was partly utilized by other bacteria. This idea is supported by the increasing cell mass during carbon dioxide-sparging. For example, some species of *Desulfovibrio* are known to use acetate as a carbon source (Badziong and Thauer, 1978).

Also, the results of the first high $p\text{CO}_2$ -experiments (Fig. 2) were somewhat different compared to those of later tests. Firstly, methane production was reduced much more: 30% of the original production compared to 67% later on. Secondly, methane production did not increase when $p\text{CO}_2$ was gradually reduced to around 0.5 atm while N_2 -sparging in the later experiments caused an immediate increase in methane production. This might have been a consequence of higher acetic acid levels (3-4 g/l) as compared to the other experiments. Another explanation might be that the methanogenic bacteria require $p\text{CO}_2 < 0.4$ atm to recover from the carbon dioxide-inhibition.

As for the mechanism for inhibition by carbon dioxide, it is likely to be a direct effect of carbon dioxide due to the immediate response in methane production to rapid changes in $p\text{CO}_2$. Accumulation of toxic compounds as a result of carbon dioxide-sparging would rather result in gradual effects on methane production. Since pH was kept at 6.8-7.0 during CO_2 -sparging by NaOH-titration sodium levels increased to 3-6 g/l. The toxic threshold was estimated to be 8 g/l (Eckenfelder, 1967). In one case, sodium levels of approximately 9 g/l were noted during CO_2 -sparging, nevertheless, methane production increased immediately when $p\text{CO}_2$ was lowered. Apparently the decreased methane production during high $p\text{CO}_2$ -conditions was not a result of decreased methanogenic cell mass. Since this would cause a gradual decrease in methane production and probably a decreased total cell mass.

When the nitrogen-atmosphere in the fermentor was changed to a methane-atmosphere with a continuous low $p\text{CO}_2$, methane production remained unchanged or increased slightly. The substrate-glucose was completely used. Although acetic acid concentrations slightly increased, they remained low (approximately 80 mg/l). The high $p\text{CH}_4$ in the gas recirculation fermentor, therefore, did not seem to have any inhibiting effect.

When a low $p\text{CO}_2$ is desired, it should be possible to sparge nitrogen or reduce the total pressure. The gas recycling carbon dioxide-absorption method reviewed here, should be practical. Its usefulness for methane production will be evaluated further.

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END PRODUCT INHIBITION IN METHANE FERMENTATIONS:
EFFECTS OF CARBON DIOXIDE ON FERMENTATIVE AND
ACETOGENIC BACTERIA

Hansson G, Molin N

SUMMARY

The rates of glucose utilization by fermentative bacteria and propionate and butyrate utilization by acetogenic bacteria were studied and their dependence of $p\text{CO}_2$ in the interval 0-1 bar was determined. A batch fermentation method was used permitting good control of fermentation parameters and rapid experiments.

The rate of glucose fermentation to acids, CO_2 and H_2 was in the order of 12,000 mg glucose/l·day which was about two orders of magnitude faster than the utilization of propionic and butyric acid by acetogenic bacteria. The rate of glucose utilization was about 30 % greater at low values of $p\text{CO}_2$ compared with 1 bar CO_2 .

Propionate degradation was strongly affected by $p\text{CO}_2$; rates were 60 mg/l·day at $p\text{CO}_2 = 1$ bar and 200 mg/l·day at $p\text{CO}_2 = 0.2$ bar. Some CO_2 was required since the rate of propionate utilization dropped rapidly below $p\text{CO}_2 = 0.2$ bar. The rate of butyric acid utilization was constant at 170 mg/l·day; slightly lower at $p\text{CO}_2 = 1$ bar.

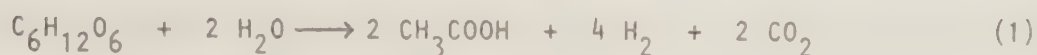
Yields of methane from glucose or acids were close to the theoretical value 50 % of degraded substrate-carbon. Yields were 20-30 % higher at low values of $p\text{CO}_2$ compared with 1 bar CO_2 .

The redox potential was usually between -200 and -250 mV, slowly increasing to between -150 and -200 mV during fermentation. No clear connection between rates of substrate utilization, $p\text{CO}_2$ and E_h was detected.

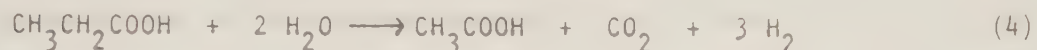
INTRODUCTION

End product inhibition is well-known in many fermentation processes and inhibition by CO_2 and/or CH_4 may be expected in methane fermentations. Three main groups of bacteria producing CO_2 or CH_4 are involved in the final stages of methane fermentations according to the following equations (Bryant 1979):

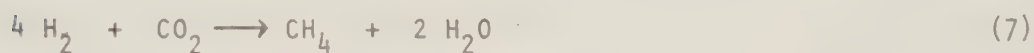
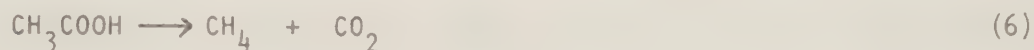
I Acetic-, propionic- and butyric acid production from carbohydrates such as glucose by fermentative bacteria:



II Oxidation of propionic acid and butyric acid by acetogenic bacteria:



III Methane production from acetic acid and H_2/CO_2 by methanogens:



Studies on end product inhibition may be performed by comparing fermentation kinetics and yields in fermentations at different levels of pCO_2 or pCH_4 . This may be achieved by carrying out fermentations at reduced or elevated pressures (Bloodgood and Anderson 1963; Mangel et al 1980; Wise et al 1978; Finney and Evans 1975). Another method is to control the gas phase by introducing CO_2 , CH_4 or inert gas in the fermenters (Keefer and Kratz 1932; Hartz and Kountz 1966; Hansson 1979). The results in the literature regarding end product inhibition are conflicting. In some cases the noted inhibiting effects are due to insufficient control of the experiments rather than CO_2 - or CH_4 -inhibition. However, it seems clear that CO_2 has inhibiting effects on some methanogens, except when grown on $H_2 + CO_2$, although the degree of inhibition at different pCO_2 is not known. No reports regarding inhibition by CH_4 have been found.

Effects of pCO_2 on fermentative and acetogenic bacteria are reported in this paper while effects of pCO_2 and pCH_4 on methanogens are reported separately (G Hansson, N Molin, accepted for publication).

MATERIAL AND METHODS

Fermentor Cultivation Technique. To determine the rate of substrate utilization at different $p\text{CO}_2$, a batch cultivation system was used (Fig. 1). Three or four fermentors (New Brunswick Multigen 2 litres) were run in parallel, being sparged with mixtures of N_2 and CO_2 to obtain certain values of $p\text{CO}_2$. One of the fermentors in each set of fermentations was sparged with pure N_2 as a standard to be able to compare rates of substrate utilization between different sets of fermentations. All gases passed through a $\text{CuO}/\text{Cu}_2\text{O}$ -column to remove traces of oxygen and were then sparged into the fermentors at approx. 1 litre/h. The fermentations were performed at 35°C and the cultures mixed using impellers at 600-700 rpm to ensure good contact between gas and liquid phase. pH was measured continuously in the fermentor with glass-calomel electrodes and controlled at 7.0 by automatic addition of 4 M NaOH or 4 M HCl. Redox potentials were measured continuously with platinum-calomel electrodes. Fermentations were started by blowing oxygen-free gas through the empty fermentor to remove all air, then inoculating with 1000 ml culture and, after equilibration, adding of 200 ml substrate.

Microbial culture. As inoculum in the fermentations a mixed culture adapted to methane production from glucose was used. This culture was originally obtained from a sewage sludge digester and had been cultivated continuously for 3 years on a glucose-based medium in a stainless steel digester. The methane-forming component in this mixed culture is probably a Methanosarcina since an increased number of Methanosarcina-clumps are observed under the microscope following cultivation on acetate. The total cell density in the inoculum culture was 3-4 g/litre.

Cultivation Media. Glucose medium (g/litre): glucose, 16; yeast extract, 4.0; NH_4Cl , 4.0; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.6; KH_2PO_4 , 0.15; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4. Trace elements (mg/litre): $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 0.60; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.66; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.18; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.18; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.14; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.14. The substances were dissolved in distilled water and the medium was boiled and allowed to cool in a N_2 -atmosphere.

Fatty acid media: The glucose was replaced by acetic-, propionic- or butyric acid or combinations of acids. Otherwise the composition and preparation were the same as for glucose medium. After substrate addition to the fermentors the total glucose/fatty acid concentration was around 3 g/l to avoid inhibition by high substrate concentrations.

Analytical Procedures. Fatty acids were analyzed by gas chromatography using a Varian model 940 chromatograph with flame ionization detector, autosampler and CDS 101 integrator. Column: 8 ft teflon column 1/16 inch by 1/8 inch with a 100 mm empty glass column at the injection end.

Support: Chromosorb 101. Carrier gas: N_2 30 ml/min, saturated with distilled formic acid. Temperatures: Injector $190^{\circ}C$, Column $175^{\circ}C$, Detector $190^{\circ}C$. Preparation of samples: Fermentation liquids are acidified, centrifuged and then injected (0.5 μ l) into the glass column. Most contaminants in the fermentation liquid will adhere to the walls of the glass column which is regularly changed to a clean column.

Gas analyses (N_2 , CH_4 , CO_2) were carried out by gas chromatography according to van Huyssteen (1967). Glucose was measured by the hexokinase method (Schmidt 1961).

RESULTS AND DISCUSSION

Effects of pCO_2 on fermentative bacteria. The fermentation of glucose to fatty acids was studied at six different levels of pCO_2 in the interval 0-1 bar. Data on the rate of glucose degradation and the distribution of glucose-carbon in products after completed fermentation of glucose (12-20 hours) is compiled in Table 1.

It is quite clear that the production of acids from glucose is much more rapid than the degradation of acids. This is in agreement with several investigations (e.g. Ghosh and Pohland 1974, Lawrence and McCarty 1969). The rate of glucose decomposition is affected by pCO_2 ; the rate is about 30 % greater in N_2 -atmosphere compared with CO_2 -atmosphere. However, since the rate of glucose fermentation is about 50-100 times faster than

acid degradation this should not be of any practical significance.

The main fatty acid products were acetic and propionic acid with only small amounts of butyric acid. The proportions of acetic and propionic acid were not significantly affected by $p\text{CO}_2$ while the amount of butyric acid increased slightly with increasing $p\text{CO}_2$. According to theories on interspecies hydrogen transfer (Wolin 1974, Zeikus 1977, McInerney and Bryant 1978) an increased production of reduced acids by fermentative bacteria should be expected when methanogenic bacteria are disturbed in a complex methanogenic system due to their impaired capacity to keep a sufficiently low $p\text{H}_2$. However, in this system $p\text{CO}_2$ affects the utilization of acetic acid by methanogens rather than their utilization of $\text{H}_2 + \text{CO}_2$ (Hansson 1979). Therefore, $p\text{CO}_2$ should not affect $p\text{H}_2$ or the product pattern of fermentative bacteria.

The amount of methane produced during the fermentative step was greatly affected by $p\text{CO}_2$ and was about 3 times greater in N_2 - compared with CO_2 -atmosphere. If, according to equations (1) and (2) above, propionic acid is found in equal or somewhat higher amount than acetic acid, not much hydrogen will be left for methane production from $\text{H}_2 + \text{CO}_2$ and therefore acetic acid will be the main precursor to methane. The differences in methane production during the fermentative step were therefore mainly due to the effects of $p\text{CO}_2$ on the rate of methane production from acetate.

During N_2 -sparging the CO_2 -production was 2-3 times higher than CH_4 -production during the initial phase of the fermentative step as was expected from the equations (1) - (3). Later on these gases were produced in almost equal amounts. Nearly all glucose-carbon in the substrate was found in products after the fermentative step during N_2 -sparging thus indicating little or no cell growth. However, some carbon from the yeast extract in the substrate may have been used. Carbon balances could be done only for N_2 -sparged fermentations since CO_2 -production could not be measured during CO_2 -sparging.

Effects of $p\text{CO}_2$ on acetogenic bacteria. The activity of acetogenic bacteria utilizing propionic and butyric acid according to equations (4) and (5) was measured as the rate of utilization of these acids at different values of $p\text{CO}_2$. In one fermentation (Fig. 2) a mixture of acetic, propionic and butyric acid was used as substrate. Another method was to use a glucose-substrate where, after the initial fermentation of glucose to acids (Table 1), the acids were utilized by acetogenic bacteria and methanogens and rates of acid utilization were calculated. Altogether, four sets of fermentation were carried out at different $p\text{CO}_2$ and data on propionic and butyric acid utilization are compiled in Table 2. Mean values, corrected for differences in inoculum quality, are shown in Fig.3.

The rate of propionic and butyric acid utilization was in the order of 100-200 mg/l·d, while the decomposition of glucose to acids was in the order of 12,000 mg/l·d. The utilization of acetic acid by methanogens was also faster than acetogenic activities.

Low rates of propionic and butyric acid utilization are expected since the thermodynamics of these reactions are unfavourable. $\Delta G'_0$ for butyrate-degradation (Eqn 4) and propionate-degradation (Eqn 5) are + 11.5 kcal/mole and + 18.5 kcal/mole, respectively, which means that very efficient hydrogen-consumption by methanogens or *Desulfovibrio* is necessary for acid-degradation to proceed (Thauer et al 1977). Consequently, the known propionate and butyrate-degrading organisms are very slow-growing with generation times of 105 h and 160 h, respectively (McInerney et al 1979, Boone and Bryant 1980). Kaspar and Wuhrmann (1978) reported propionate degradation rates of 50 mg/l·d in digested sludge and 340 mg/l·d when the sludge was saturated with propionate.

Butyrate-degradation was not much affected by $p\text{CO}_2$, and only a slight inhibition occurred at 1 bar CO_2 . This was expected since no CO_2 is formed according to equation 5. In propionate-degradation however, CO_2 is formed and reaction rates are also significantly affected by $p\text{CO}_2$. Propionic acid utilization was 3-4 times faster in 20 % CO_2 than in 100 % CO_2 .

Evidently some CO_2 was required since the rate dropped rapidly below 20 % CO_2 and was as low in N_2 -atmosphere as in CO_2 -atmosphere.

These results suggest that in methane production from glucose the degradation of propionic and butyric acid are the slowest steps. At normally occurring values on $p\text{CO}_2$ (0.4-0.5 bar) the rates of propionate and butyrate utilization are about the same but at high values of $p\text{CO}_2$ the rate of propionate utilization drops rapidly. The rate also drops below $p\text{CO}_2=0.2$ bar but this situation should not occur in practical applications.

In a well-working digester the fermentation is pulled towards acetate-production due to the mechanism of interspecies hydrogen transfer. The problem of propionate and butyrate degradation would therefore be most important in a fermentation where a disturbance has led to increased concentrations of these acids or in situations where the normal decomposition of substrates proceeds with reduced acids as intermediates. However, in methane-production from complex materials the initial hydrolytic and fermentative steps may be rate-limiting rather than acid degradation (Hobson et al 1974).

Yields of methane. Total gas yields ($\text{CH}_4 + \text{CO}_2$) can be calculated only in N_2 -sparged fermentors since otherwise external CO_2 is introduced into the fermentors. Therefore methane yields have been calculated for all fermentations on glucose and mixed acid substrates. Yields are expressed as percentage of substrate-carbon degraded to methane. Yield data from 13 fermentations are shown in Fig. 4. In most fermentations complete degradation of acids was accomplished within 5-12 days.

Theoretically, CO_2 - and CH_4 -yields from glucose are 50 % each (Buswell and Mueller 1952). This is in agreement with our results at low values of $p\text{CO}_2$. Yields were even slightly higher than 50 % but some methane formation may be due to decomposition of cell mass and yeast extract. Methane yields were decreased with increasing $p\text{CO}_2$ and at 1 bar CO_2 yields were around 40-45 %, i.e. the difference in methane yields at high and low $p\text{CO}_2$ were 20-30 %.

On mixed acid substrates the theoretical methane yield is also 50 %. In this study the yields and their dependence on $p\text{CO}_2$ were the same or slightly higher compared with yields on glucose substrate. In N_2 -atmosphere, however, the methane yield dropped as the rate of propionate utilization dropped as discussed above.

Redox potential measurements. The redox potential (E_h) was measured continuously in all batch fermentations. The objective was partly to detect any connections between redox potential and gas phase composition or substrate consumption rate, partly to monitor the general condition of the culture and detect sudden changes due to disturbances.

In general, after inoculation and substrate addition, the redox potential rapidly dropped to between -200 and -250 mV (actual readings were around 238 mV less due to the potential of the reference calomel electrode). This was the case in all gas atmospheres: N_2 , CO_2 or N_2/CO_2 -mixtures. The redox potential then usually increased slowly to between -150 and -200 mV but in some cases it remained rather constant, in other cases, especially at high $p\text{CO}_2$, it increased rapidly to -100 mV towards the end of the fermentation. Since there was no clear connection between rates of substrate utilization, $p\text{CO}_2$ and E_h , the mechanism by which CO_2 affects methane-producing systems does not seem to involve the redox potential. The observed redox potentials in this work, -150 to -250 mV correspond to electrode potentials of -388 to -488 mV. According to the literature methane fermentations work well at electrode potentials below -360 mV (Smith and Hungate 1958).

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Table 1. Rate of glucose degradation and distribution of fermentation products as a function of gas composition

	N_2	20% CO_2	40% CO_2	60% CO_2	70% CO_2	CO_2
Glucose degradation mg/l·h	552	504	503	508	504	415
% of glucose carbon in products						
HAc	15	14	26	18	25	20
HPr	33	33	19	31	33	24
HBut	0.5	1.8	0	6	3.2	8
CH_4	18	18	13	9	10	6
CO_2	34	-	-	-	-	-
Total	100					

Table 2. Rates of propionic and butyric acid degradation in 17 fermentations on glucose or mixed acids

	Expt no	N ₂	20% CO ₂	30% CO ₂	40% CO ₂	60% CO ₂	70% CO ₂	CO ₂
HPr mg/l·d	I	47		150		113		59
	II	77				157		94
	III	54	205				114	
	IV	55			190			63
HBut mg/l·d	I			166		170		138

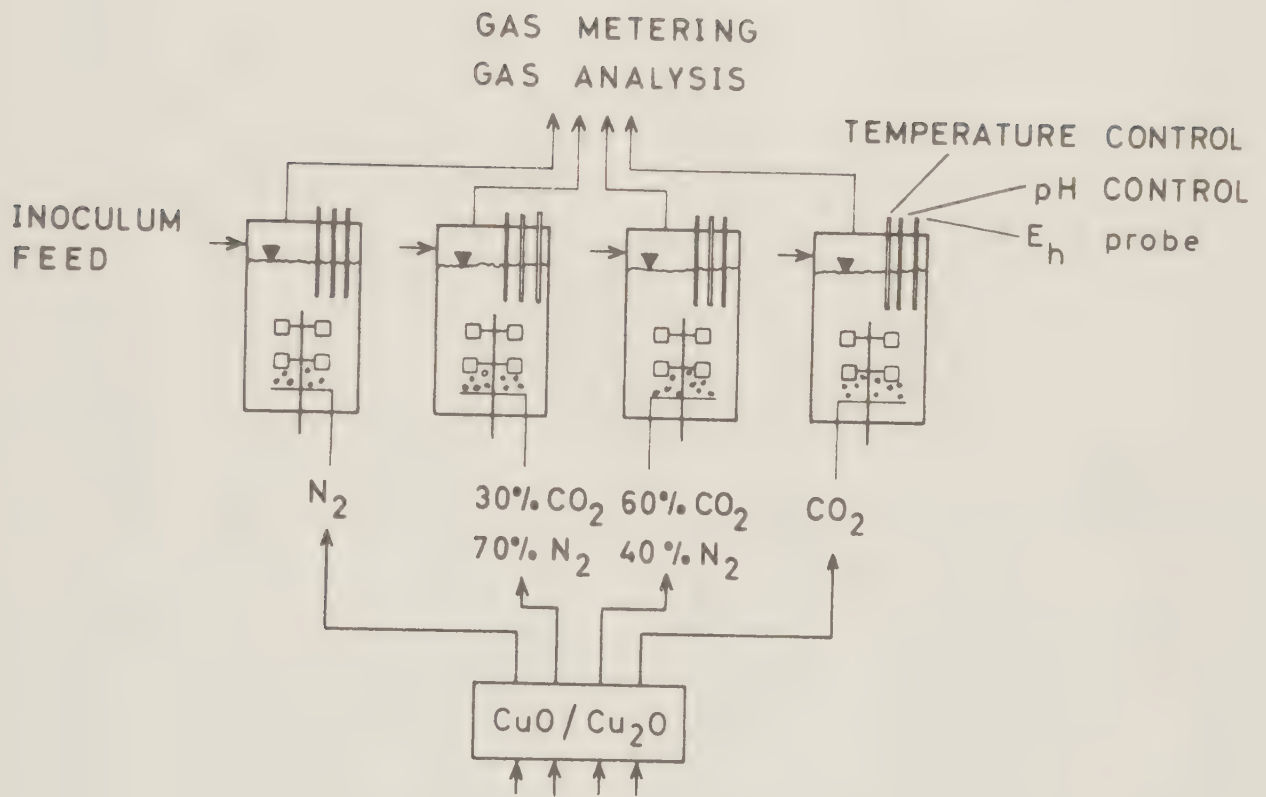


Fig. 1 Schematic diagram of fermentor panel for batch fermentations at different $p\text{CO}_2$.

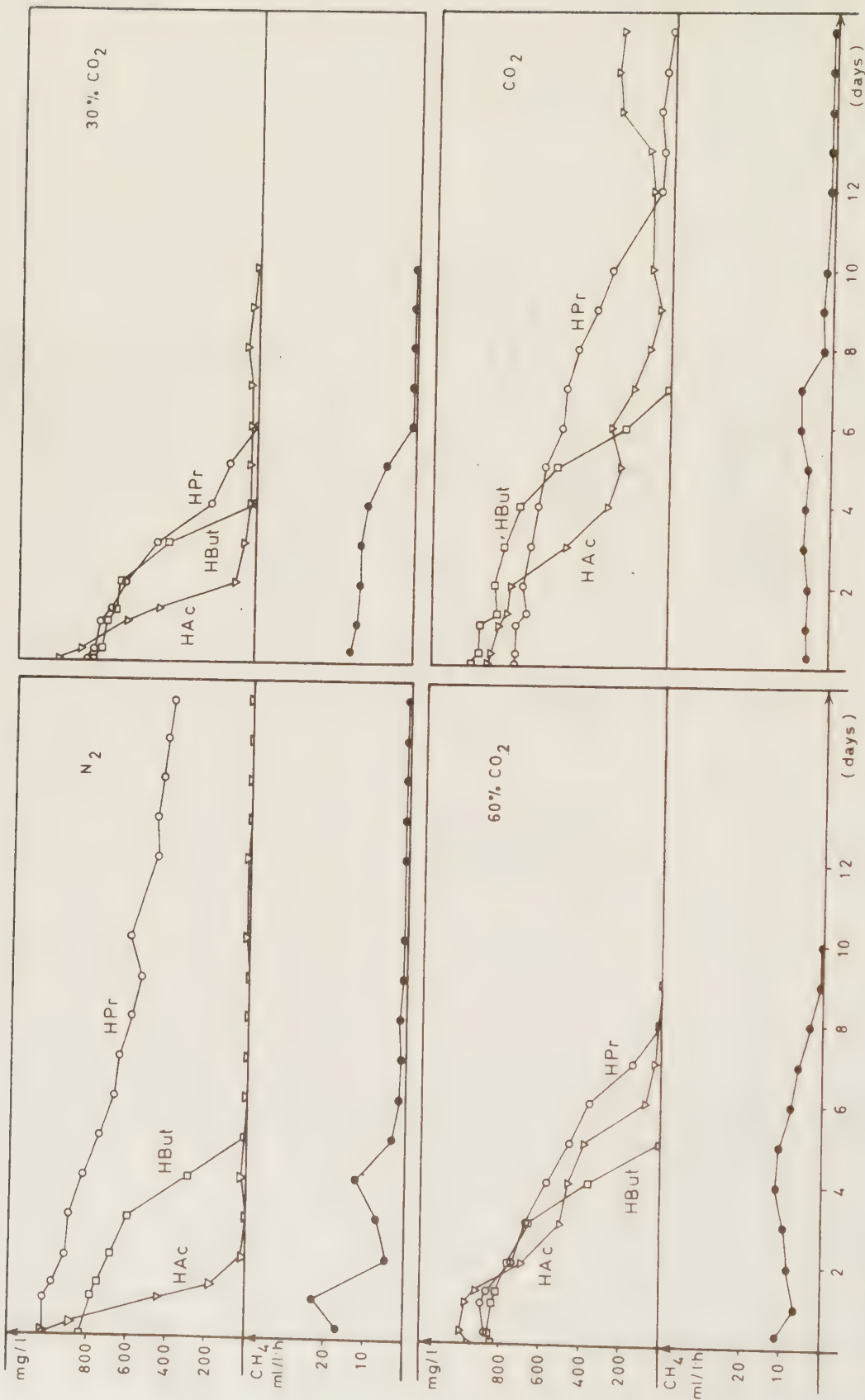


Fig. 2 Fermentation of a mixture of acetic, propionic and butyric acid in N₂, 30 % CO₂, 60 % CO₂ and CO₂.

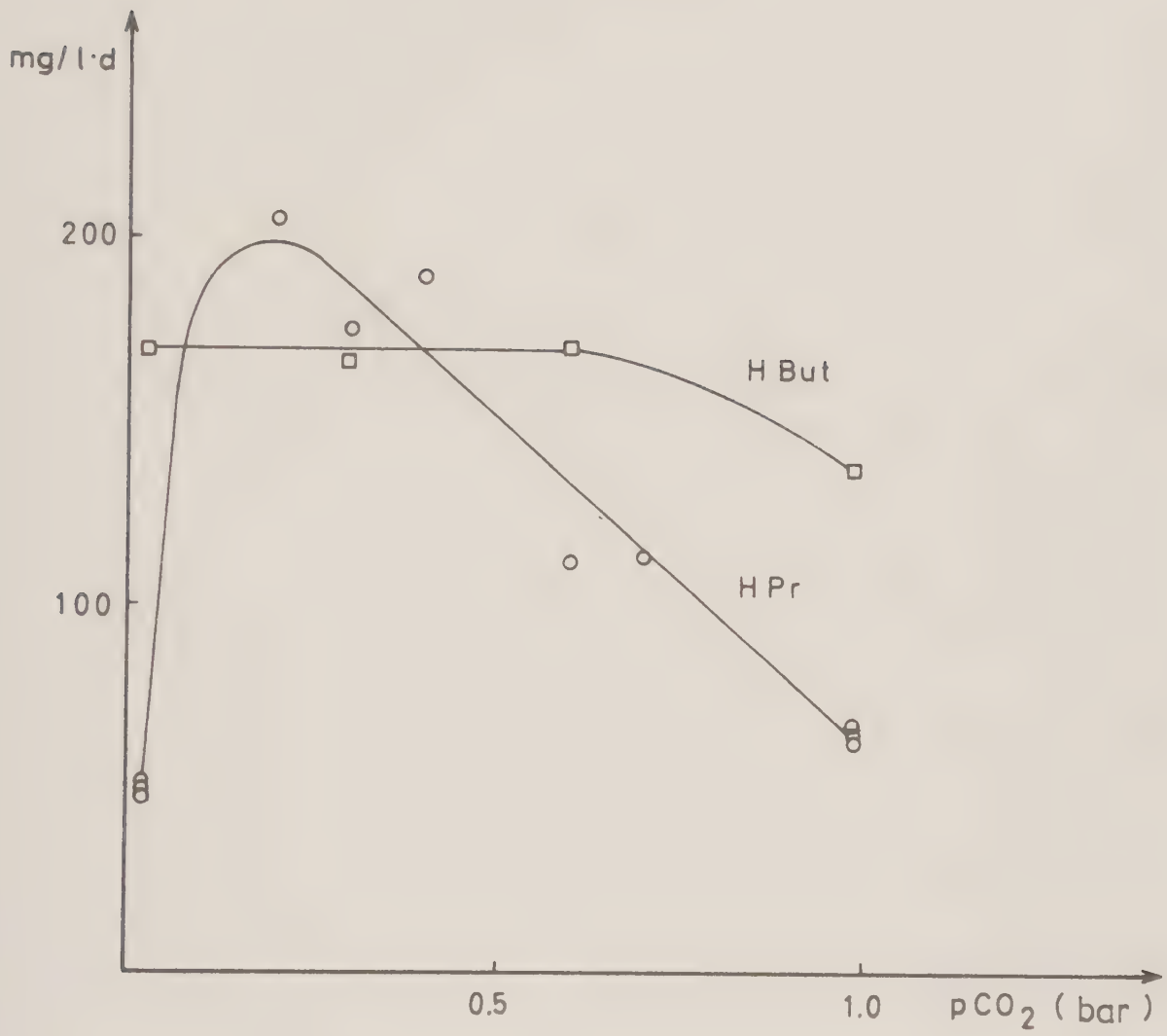


Fig. 3 Rates of acetogenic reactions as a function of pCO₂.

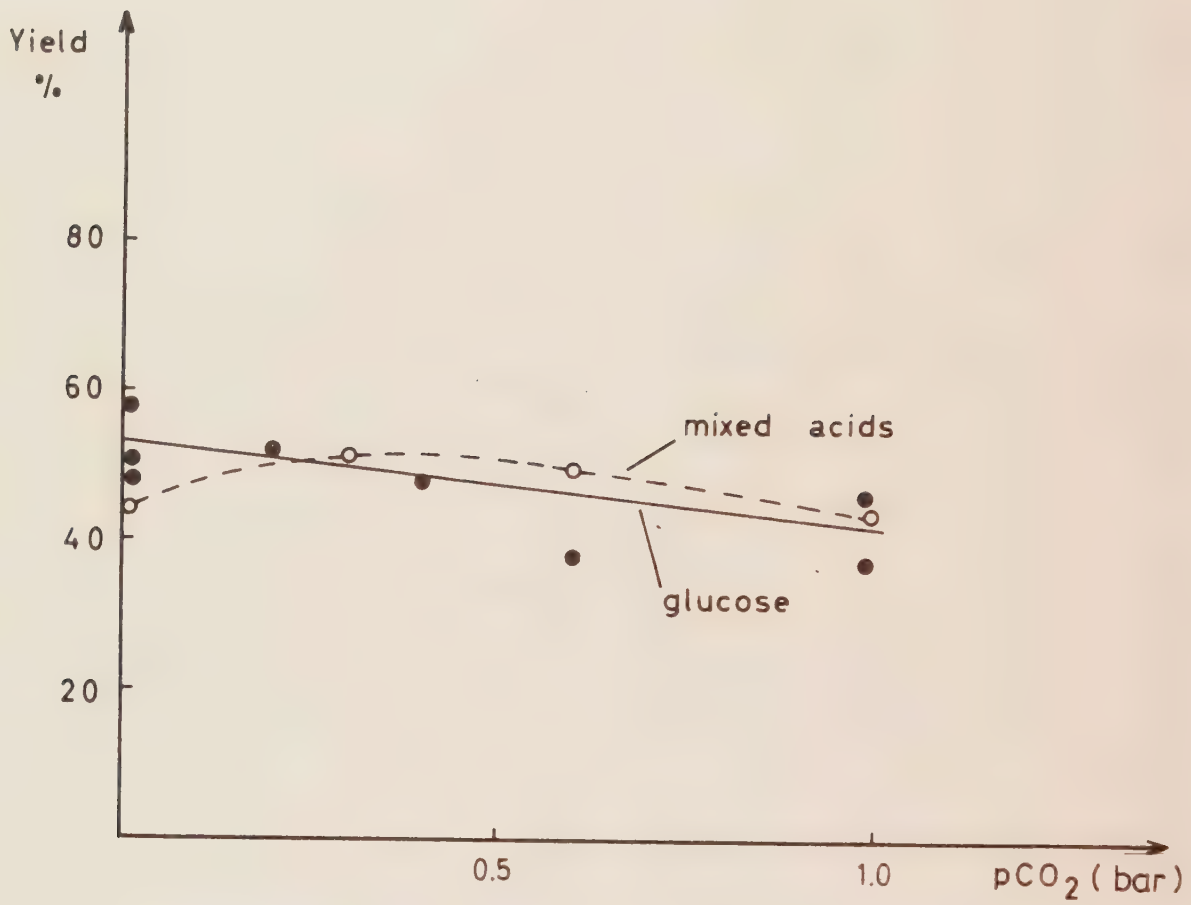


Fig. 4 Yields of methane on glucose and mixed acid substrates at different pCO_2 .

III

END PRODUCT INHIBITION IN METHANE FERMENTATIONS:
EFFECTS OF CARBON DIOXIDE AND METHANE ON METHANOGENIC
BACTERIA UTILIZING ACETATE

Hansson G, Molin N

SUMMARY

The effects of $p\text{CO}_2$ and $p\text{CH}_4$ in the interval 0-1 bar on rates of acetate degradation and methane formation by methanogens as well as methane yields were studied in enrichment cultures in batch and continuous fermentations.

In batch fermentations the rate of acetate utilization by methanogens was 1000-1500 mg/l·d at low levels of $p\text{CO}_2$. CO_2 was inhibitory and degradation rates were around 350 mg/l·d in 1 bar CO_2 . The degradation of acetate was almost linear. In continuous culture maximum rates of acetate utilization around 2500 mg/l·d were obtained and the acetate concentration in the substrate was reduced by 98-99 %.

The yields of methane on acetate substrates were close to the theoretical value (1 mole CH_4 per mole HAc) in the interval $p\text{CO}_2 = 0-0.5$ bar. In 1 bar CO_2 yields decreased by 20-30 %.

CH_4 was found to be only slightly inhibitory; the inhibiting effects of 1 bar CH_4 on acetate degradation rates were comparable to the effects of 0.3 bar CO_2 . Also gas sparging and rapid mixing had small effects compared with a non-sparged, slowly mixed culture.

The redox potential was usually around -200 mV during fermentations and no connection was found between acetate degradation rate, E_h and $p\text{CO}_2$.

Acetate and propionate degradation were the reactions most sensitive to $p\text{CO}_2$ and to obtain maximum rates as well as maximum methane yields $p\text{CO}_2$ -levels around 0.2 bar were found to be optimal.

INTRODUCTION

A common feature to all methanogens known in pure culture is their ability to grow on hydrogen and carbon dioxide (Balch et al 1979). However, in methane fermentations in natural environments and waste treatment acetate is a key intermediate and may be the precursor of 70-75 % of the methane formed (Smith and Mah 1966; Jeris and McCarty 1965) while $\text{H}_2 + \text{CO}_2$ account for the rest. Although the decomposition of acetate is of great importance,

little is still known about acetate-utilizing methanogens. Many attempts to obtain acetate-utilizing methanogens in pure culture have failed or acetate decomposition has been extremely slow due to the low energy yields, -7.4 kcal/mole, in acetate-splitting. Only two species have so far been described to use mainly acetate in pure culture (Smith et al 1980). One, Methanosarcina barkeri, may use methanol and methylamines as well as acetate. The other, a rod-shaped or filamentous bacterium (so far called the "acetate organism" or the "fat rod"), was recently isolated by Zehnder et al (1980) and appears to differ from all other methanogens in its inability to use $H_2 + CO_2$.

The methanogenic step is generally regarded as rate-limiting in methane production from soluble substrates (Lawrence and McCarty 1969). Acetate degradation is the crucial reaction since most methane is formed this way and methane formation from $H_2 + CO_2$ is considerably faster as demonstrated by Wise et al (1978). Therefore, knowledge on cultivation parameters that may stimulate or inhibit methanogens and in particular acetate utilization by methanogens are important. Two such fermentation parameters are the partial pressures of the product gases, pCO_2 and pCH_4 , and the possibility of end product inhibition. Our previous work (Hansson 1979) indicated inhibiting effects of CO_2 but not CH_4 on methane production from glucose. In this paper the effects of pCO_2 and pCH_4 in the interval 0-1 bar on acetate utilization by methanogens are reported. Effects of pCO_2 on fermentative and acetogenic bacteria are reported separately (Hansson and Molin, accepted for publication).

MATERIAL AND METHODS

Batch cultivations. The activity of methanogens was measured as rate of acetate utilization and methane production in highly mixed batch cultures at 35° C. Two or three fermentors were run in parallel, one with N_2 -sparging as a standard, the others with mixtures of N_2 and CO_2 , pure CO_2 or pure CH_4 to obtain values of pCO_2 and pCH_4 in the interval 0-1 bar.

Details of this technique and of the used microbial culture are

discussed elsewhere (Hansson and Molin, accepted for publication).

Continuous cultivations. Continuous cultures on acetate media were performed in 2 litre New Brunswick fermentors, effective volume 1 litre. The temperature was 37° C and the culture was mixed by impellers at 600 rpm. pH was measured continuously with glass-calomel electrodes and controlled at 7.0 by automatic addition of 4 M NaOH or 4 M HCl. N₂ was passed through a CuO/Cu₂O column at 360° C to remove traces of oxygen and then sparged through the fermentor at a rate of around 2 litre/h. The retention time was 6-14 days and the loading rate of acetate between 1.2 and 2.4 g/litre, day.

Cultivation Media. Acetate medium (g/litre): Na-acetate, 22; yeast extract, 4.0; NH₄Cl, 4.0; Na₂HPO₄·2H₂O, 1.6; KH₂PO₄, 0.15; MgSO₄·7H₂O, 0.4. Trace elements (mg/litre): FeCl₃·6H₂O, 0.60; CaCl₂·2H₂O, 0.66; ZnSO₄·7H₂O, 0.18; CoCl₂·6H₂O, 0.18; CuSO₄·5H₂O, 0.14; MnSO₄·4H₂O, 0.14.

The substances were dissolved in distilled water and the medium was boiled and allowed to cool in a N₂-atmosphere. In media for continuous cultivation acetate was added as HAc after sterilization.

Analytical Procedures. Fatty acid analysis was carried out by a gas chromatographic method described elsewhere (Hansson and Molin, accepted for publication).

Gas analysis (N₂, CH₄, CO₂) were carried out by gaschromatography according to van Huyssteen (1967).

RESULTS AND DISCUSSION

Effects of pCO₂ on acetate utilization in batch fermentations. The degradation of acetate and production of methane was studied at six different levels of pCO₂. Seven sets of fermentations were carried out and data on acetate degradation rates are compiled in Table 1. The acetate consumption rates were 1000-1500 mg/l·d in N₂-atmosphere with the mean value 1300 mg/l·d. The reasons for these differences were probably variations in the

activity of the inoculum culture which in turn may be due to fluctuations in the liquid level and gas phase composition in the inoculum fermentor. However, to make values of acetate degradation rates in different fermentation experiments comparable all figures are related to the mean rate of acetate degradation, 1300 mg/l.d. The resulting curve, showing the influence of $p\text{CO}_2$ on acetate degradation, is shown in Fig. 1.

Our rate of acetate degradation compares fairly well with literature data. In pure cultures of Methanosarcina barkeri Weimer and Zeikus (1979) found a degradation rate of 112 mg/l.d, in both cases in cultures equilibrated with N_2 . The rod-shaped "acetate organism" (Zehnder et al 1980) consumed acetate at 90 mg/l.d at $p\text{CO}_2$ approximately 0.2 bar. In acetate enrichments van den Berg et al (1976) obtained acetate consumption rates of 300-600 mg/l.d ($p\text{CO}_2$ approx. 0.4 bar) and Baresi et al (1978) found 480 mg/l.d in N_2 -atmosphere without shaking. Very high values of acetate degradation are reported after spike injections of acetate in digestors fermenting sewage sludge and cellulose. Kaspar and Wuhrmann (1978) reported acetate degradation rates of 600 mg/l.d, Stafford et al (1980) found 960-3400 mg/l.d and Winter and Cooney (1980) approximately 4000 mg/l.d.

Data on acetate degradation rates in the literature are very varying due to used microbial culture, cultural conditions, media etc. Previous data were therefore not usable for evaluating the possible influence of CO_2 on acetate degradation. Many of the reported acetate-degrading fermentations were not carried out at constant $p\text{CO}_2$ since, due to the gas evolution, $p\text{CO}_2$ will increase during the experiment. Our own work carried out under controlled conditions, suggests a strong inhibiting effect of $p\text{CO}_2$ with a four-fold difference in acetate degradation rate at $p\text{CO}_2 = 1$ bar compared to low values of $p\text{CO}_2$.

Redox potential measurements. The redox potential, E_h , was measured continuously in all batch fermentations, partly to detect any connections

between E_h , pCO_2 and acetate utilization rate and partly to monitor the general condition of the culture.

In N_2 -atmosphere E_h usually remained at approximately -200 mV during the fermentations (actual reading around -440 due to the potential of the reference calomel electrode). In N_2/CO_2 -mixtures E_h usually started to increase slowly when the fermentation of acids was almost completed. In the two fermentations in pure CO_2 (Table 1) E_h behaved somewhat differently. In Expt no I E_h remained below -200 mV during the whole fermentation while in Expt no VI E_h increased to -100 mV after two days so most of the fermentation was carried out at -80 to -100 mV. This had no effect, however, on the acetate degradation rate and may have been due to electrode error. Generally, no clear connection between E_h , pCO_2 and acetate degradation rates were detected.

Effects of pCO_2 on methane yields. In methane production from acetate almost 1 mole of CH_4 is produced from 1 mole of acetate and only small amounts of acetate are oxidized or assimilated (Smith et al 1980). In Fig. 2 accumulated amounts of degraded acetate and produced methane versus time is shown in fermentations with the gas phase N_2 , 70 % CO_2 and CO_2 , respectively. In all three fermentors acetate degradation and methane production were almost linear indicating that no substantial growth of acetate-utilizing methanogens occurred. Complete degradation of acetate was finished after 2.3, 4.1 and 9.3 days, respectively. In N_2 -atmosphere methane production continued slowly after acetate utilization was completed. Presumably, this methane was produced from the yeast extract in the substrate or from cell components liberated by lysis of fermentative and acetogenic bacteria from the inoculum.

Yields of methane were calculated as total mmoles of methane produced (after complete acetate degradation) per total mmoles of acetate degraded, $Y_{CH_4/HAC}$. Yield data for 12 fermentations are shown in Fig. 3. Due to experimental difficulties in measuring methane production in sparged fer-

mentors, yield data are rather uncertain at low values of $p\text{CO}_2$ when the methane content in the gas may change rapidly. $Y_{\text{CH}_4/\text{HAc}}$ was around 1.0 in the interval $p\text{CO}_2 = 0-0.5$ bar and then started to decrease with increasing $p\text{CO}_2$. At 1 bar $p\text{CO}_2$ the methane yield had decreased by 20-30 %.

Continuous methane production from acetate in N_2 -atmosphere. Three continuous cultivations were carried out with similar results. A fermentation diagram from one continuous culture is shown in Fig. 4. The inoculum, which was previously cultured on glucose, was capable of immediate and almost complete degradation of acetate at a loading rate of 1.2 g/l·d. Microscopic observations revealed a gradual increase in number and size of Methanosarcina-clumps. The acetate-concentration in the substrate was 12000-16000 mg/l and 98-99 % of the acetate was fermented to equal amounts of CH_4 and CO_2 . Maximum values of acetate degradation were around 2500 mg/l·d and maximum methane production around 1050 ml CH_4 /l·d at a loading rate of 2.4 g HAc/l·d. Some methane may have been formed from yeast extract or cell components. The sudden failure of the fermentation after 35 days was probably due to air leakage into the fermentor in combination with the gradual increase in the number of oxygen-sensitive bacteria in acetate enrichments. The temporary decrease in acetate level during day 36-38 was due to pump failure causing reduced substrate input. A slow but steady production of propionic acid started after 1 week, which is surprising on an acetate medium. Propionic acid may be produced in degradation of yeast extract or cell components or by reduction of acetate. During day 22-23 the gas yield was around 110 % (100 % = 1 mole CH_4 + 1 mole CO_2 from 1 mole acetate) and at the same time acetic and propionic acid accumulated, indicating that other substrate components, besides acetate, must have been utilized.

Effects of $p\text{CH}_4$ and sparging/no sparging on acetate utilization. One set of fermentations was carried out with N_2 -sparging, no gas-sparging combined with reduced mixing and CH_4 -sparging. All fermentors were sparged empty with N_2 before filling with inoculum and substrate. Rates of

acetate utilization are shown in Fig. 5. The mean rates of acetate degradation were 1390, 1160 and 1100 mg/l·d in N_2 , no sparging and CH_4 , respectively. Consequently, CH_4 was found to be considerably less inhibiting on acetate utilization than CO_2 . This is in agreement with our earlier work in continuous culture (Hansson 1979), where no inhibiting effects of CH_4 were found.

In the fermentation without sparging and reduced mixing the gas phase was initially N_2 ; then pCO_2 and pCH_4 increased and most of the fermentation was carried out in 30 % CO_2 and 40-50 % CH_4 . The lower value of pCO_2 should be due to dissolving of CO_2 as bicarbonate. Any increase or decrease in acetate utilization in this experiment may be due to either no sparging/reduced mixing, or $pCO_2 = 0.3$ bar or $pCH_4 = 0.4-0.5$ bar (the latter should not be significant). The rate of acetate degradation was approximately the same as in a sparged and highly mixed fermentation with 30 % CO_2 . Therefore, the mixing and gas sparging carried out in the fermentations do not seem to affect the rate of acetate utilization.

CO_2 -effects on rates of acetate degradation compared to degradation of glucose, propionate and butyrate. In Fig. 1 the effects of pCO_2 on rates of acetate utilization by methanogens are compared with corresponding effects on glucose degradation by fermentative bacteria and propionate and butyrate degradation by acetogenic bacteria as reported separately (Hansson and Molin, accepted for publication). The great difference in substrate degradation rates between the groups of bacteria should reflect the thermodynamics of the corresponding reactions. $\Delta G_o'$ for glucose degradation to acids is around -60 kcal/mole, for acetate degradation -7.4 kcal/mole, for butyrate degradation +11.5 kcal/mole and for propionate degradation +18.5 kcal/mole (Thauer et al 1977, McCarty 1971). Although these reactions are not carried out during standard conditions and especially the acetogenic reactions are pulled to make the reactions possible the observed reaction rates are consonant with the thermodynamics.

CO_2 affected the rates of glucose-, acetate- and propionate-degradation but only to little extent butyrate-degradation. Since glucose degradation was considerably faster than acid degradation irrespective of pCO_2 the most important effects of CO_2 concern acetate and propionate degradation. To obtain maximum rates of acetate degradation pCO_2 should be as low as possible while maximum propionate degradation was found at pCO_2 around 0.2 bar. Also the highest yields of methane on glucose or acid substrates were found at low levels of pCO_2 . Therefore, pCO_2 -values around 0.2 bar seem to be optimum for rates of acid degradation and methane yields while pCH_4 up to 1 bar had no substantial inhibiting effects.

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Table 1. Rates of acetate degradation in 15 batch fermentations at different levels of pCO_2

Approx. % CO_2 in gas phase	2	23	31	41	70	99
Acetate degradation mg/l·d in Expt. no						
I	1150					292
II	1520			1166		
III	1465	1342				
IV	1234		961			
V	1018				600	
VI	1140				756	301
VII	1550	1188				

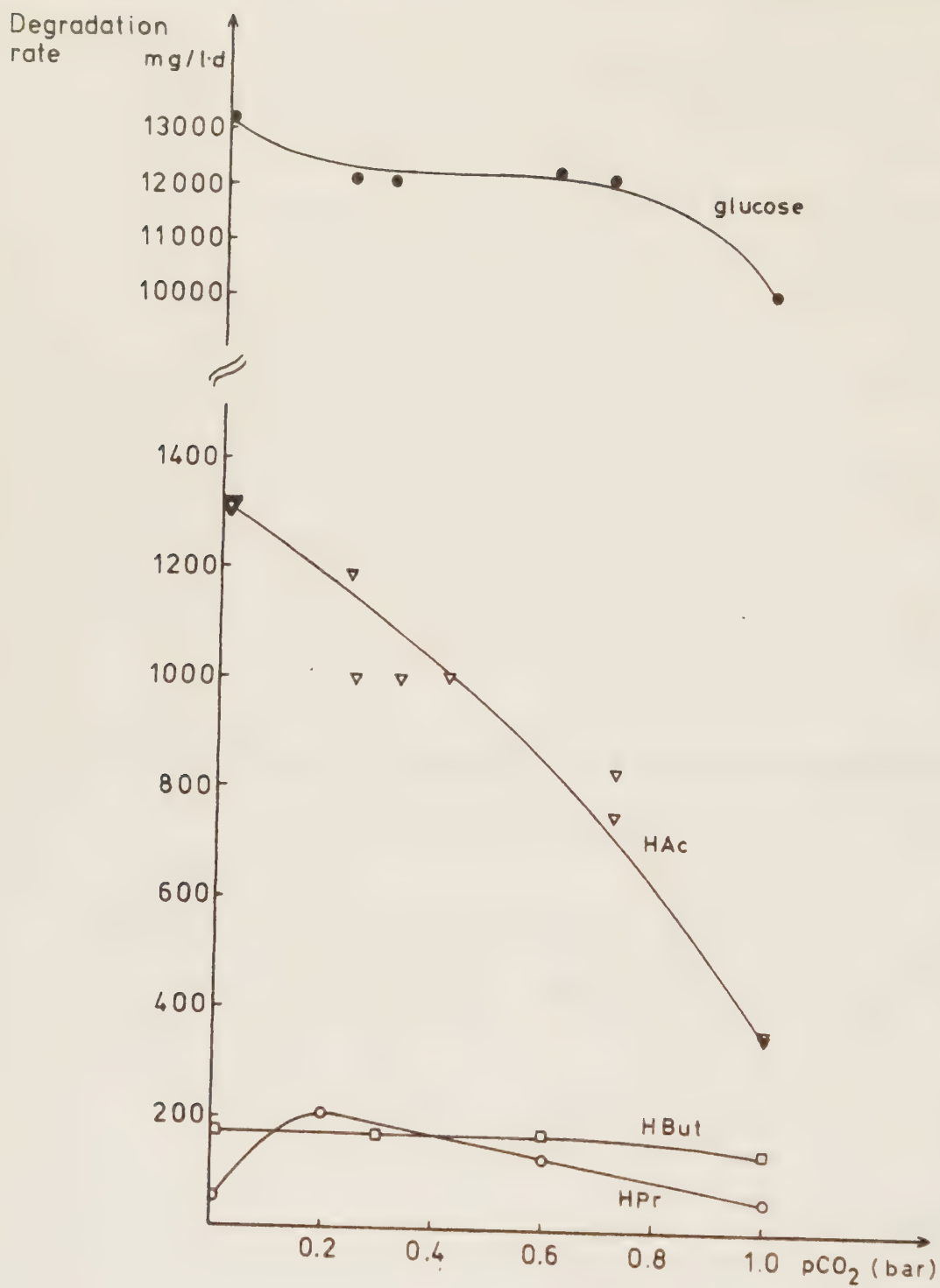


Fig 1. Effects of $p\text{CO}_2$ on the degradation rates of glucose, acetate, propionate and butyrate.

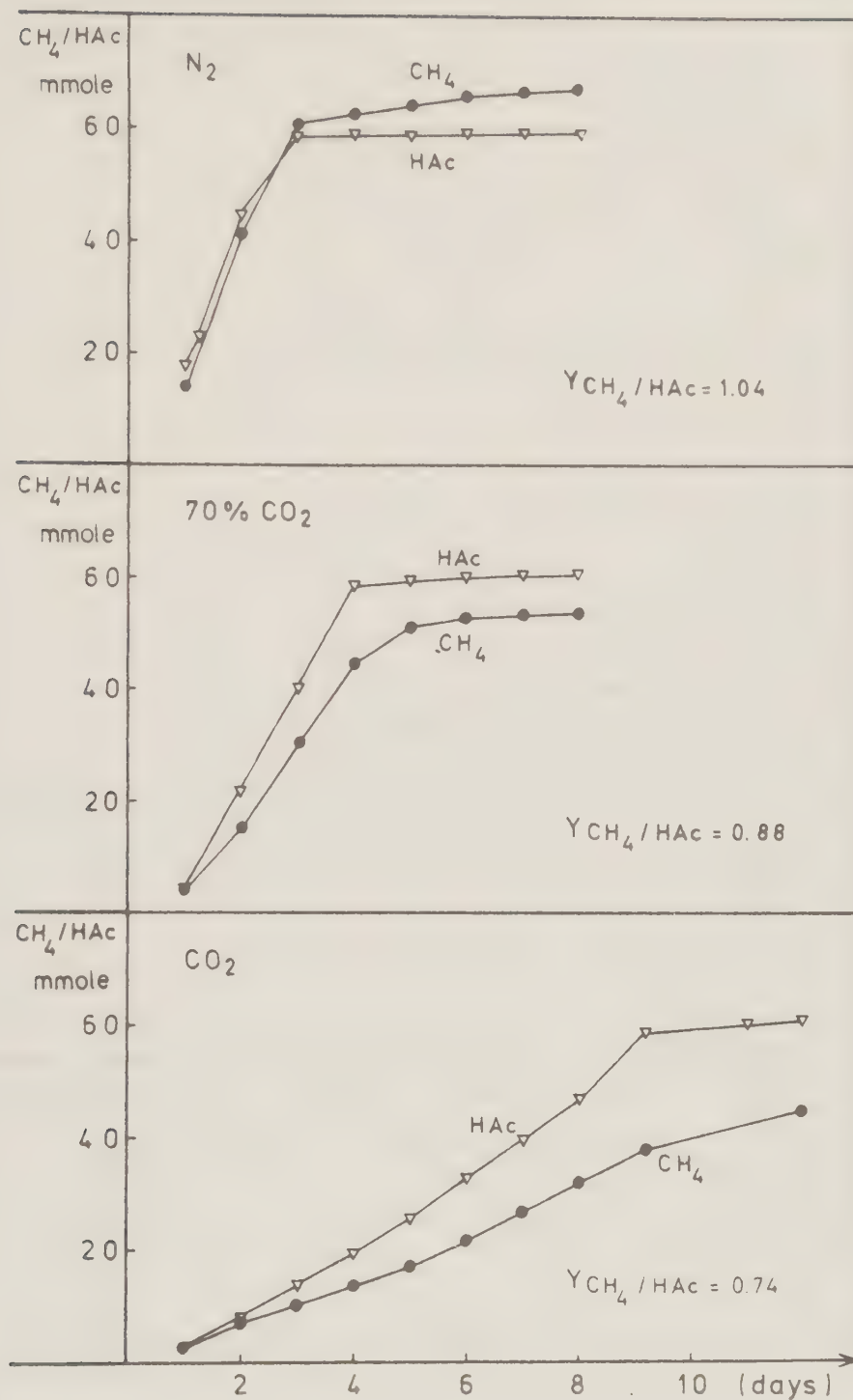


Fig. 2 Accumulated amounts of degraded acetate and produced methane in N₂, 70 % CO₂ and CO₂.

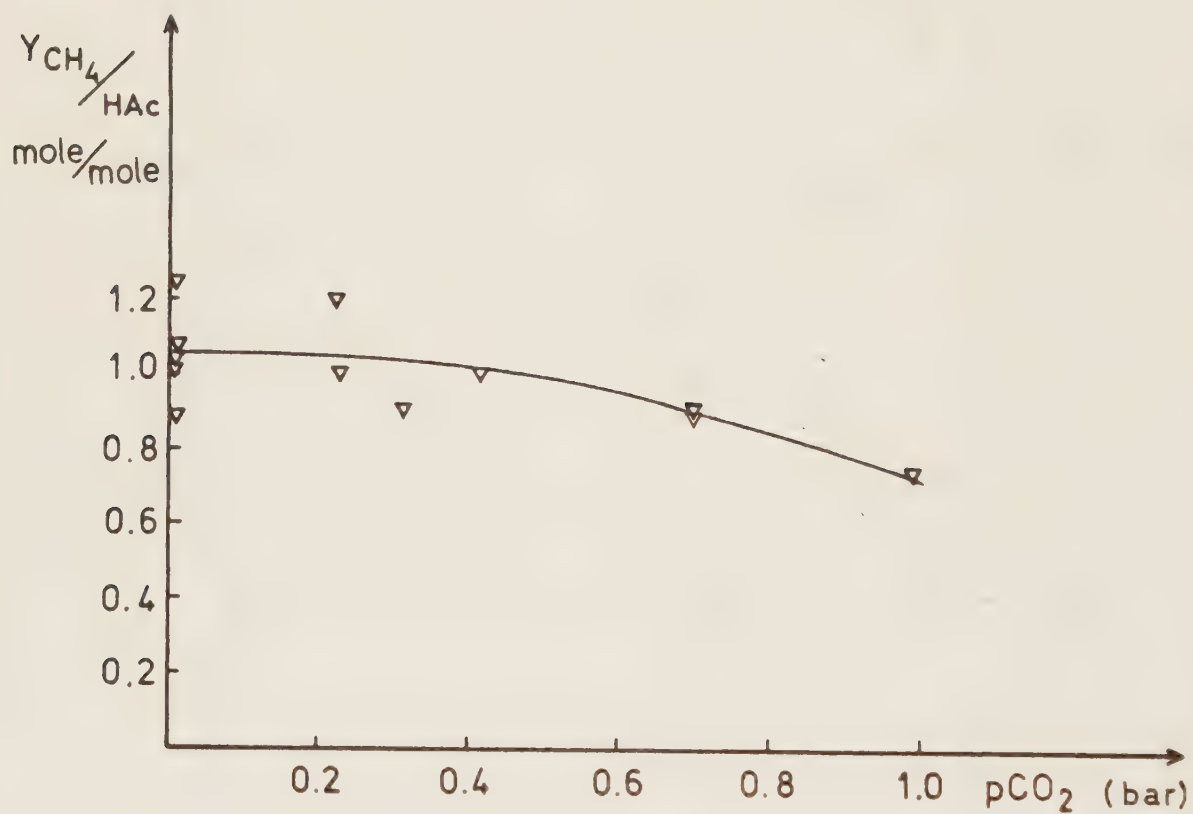


Fig. 3 Yields of methane produced from acetate at different pCO₂.

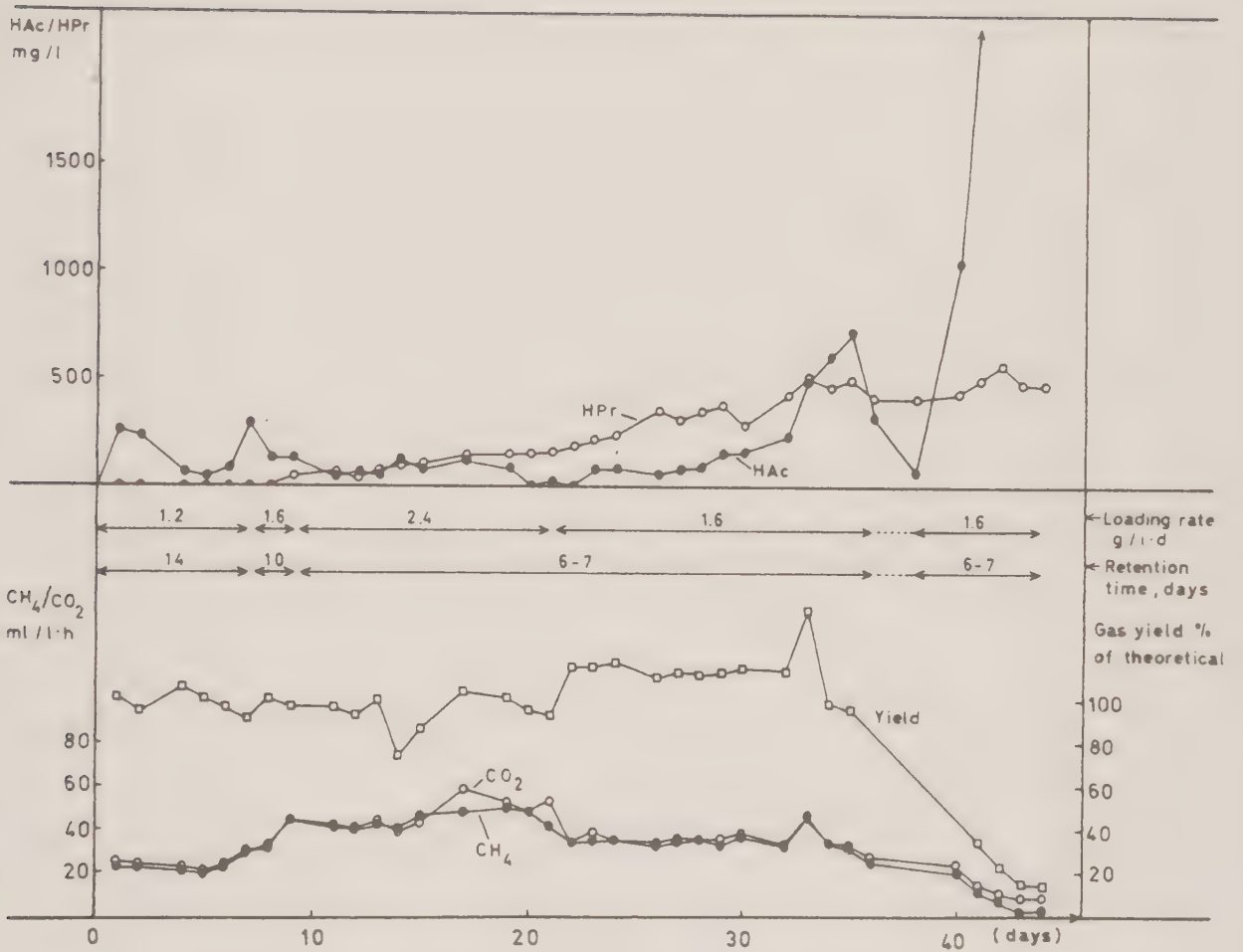


Fig. 4 Methane production from acetate in continuous culture in N₂-atmosphere.

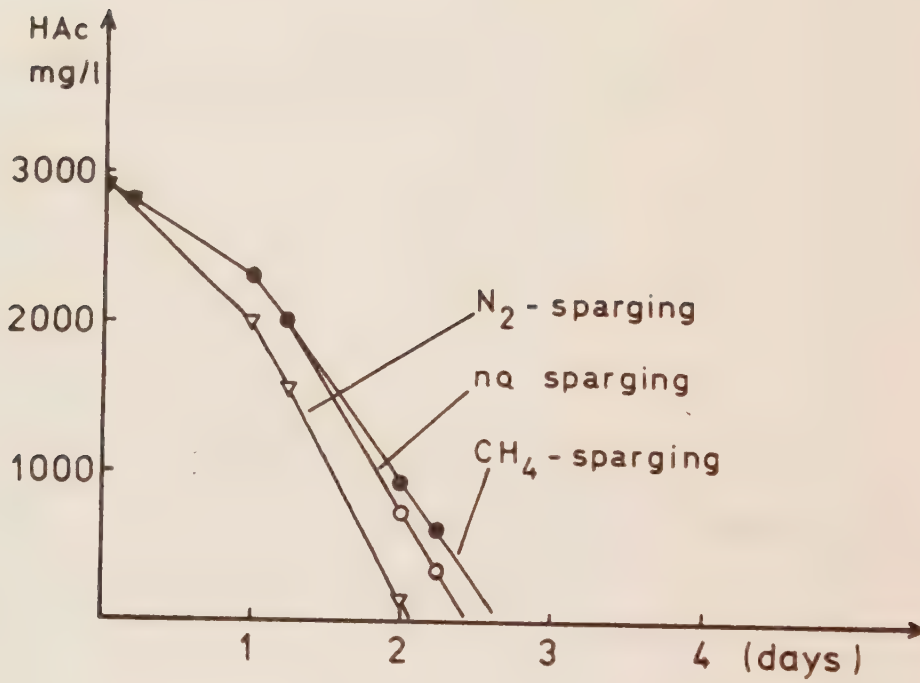


Fig. 5 Effects of 1 bar CH₄ and no sparging/reduced mixing on the rate of acetate degradation.

IV

METHANE PRODUCTION FROM GLUCOSE AND FATTY ACIDS

AT 55-85°C: ADAPTION OF CULTURES AND EFFECTS OF $p\text{CO}_2$

Hansson G

SUMMARY

Thermophilic cultures producing methane from glucose at 55°C were initiated with inocula from a mesophilic glucose enrichment, mesophilic sewage sludge, a thermophilic sugar-beet waste culture and this culture stored at room temperature for 5 months. In all cases thermophilic methane production started immediately and after 3-14 days all cultures used the substrate completely without acid accumulation. The results indicate that the mesophilic bacteria had the capacity to rapidly switch to thermophilic conditions. After one week of adaption of the mesophilic glucose-enrichment to 55°C acetate and butyrate were degraded at about the same rates as in the original culture but propionate-degrading organisms did not adapt to 55°C. Yields of methane from acid degradation were significantly lower during thermophilic compared to mesophilic conditions.

Effects of combinations of pCO_2 (0-1 bar) and temperature (55-70°C) on rates of acetate degradation were studied. Rates were 3-4 times faster at low pCO_2 than high pCO_2 . Temperature maximum was higher in low pCO_2 -environment indicating that CO_2 -like temperature- affects cell membranes.

In a glucose-enrichment in N_2 -atmosphere the temperature was gradually increased up to 85°C. Methane yields decreased with increasing temperature but were still 20-30% of theory at 80°C. At 85°C fermentation of glucose stopped and only traces of methane were produced.

INTRODUCTION

Methane production at thermophilic temperatures is receiving increased attention. Among possible advantages are faster reaction rates, improved solids - liquid separation and increased destruction of pathogenic organisms while the high energy requirements for heating and possibly poorer process stability and higher COD in effluents are disadvantages (Buhr and Andrews 1977, Varel et al 1977). Usually, in digestion of organic wastes, temperatures between 50°C and 60°C are employed and gas production is considerably lower at 65°C (Buhr and Andrews 1977, Ward 1978).

The microbiology of thermophilic methane production is poorly understood. Only two thermophilic methanogens are presently known in pure culture.

Methanobacterium thermoautotrophicum is an autotrophic, extreme thermophile growing on $H_2 + CO_2$ only (Zeikus and Wolfe 1972). Its temperature optimum is $65-70^{\circ}C$ and maximum $75^{\circ}C$. Zinder and Mah (1979) isolated a thermophilic strain of Methanosarcina utilizing acetate, methanol and methylamines but not $H_2 + CO_2$. Temperature optimum was $50^{\circ}C$ and maximum between $55^{\circ}C$ and $60^{\circ}C$. Since acetate is regarded as the major precursor of methane in digestion of organic material (Smith and Mah 1966, Jeris and McCarty 1965), it thus appears that the temperature maximum for acetate conversion to methane in pure as well as complex cultures, is around $65^{\circ}C$. Autotrophic methane production from $H_2 + CO_2$ however, may occur in the extreme thermophilic range (Zeikus 1979).

Inhibiting effects of CO_2 on methanogenic as well as fermentative and acetogenic bacteria have been studied in mesophilic cultures (Hansson 1979, Hansson and Molin 1981 a, 1981 b). In this paper the adaption of mesophilic cultures to thermophilic conditions and the effects of pCO_2 in the temperature range $55-85^{\circ}C$ are reported.

MATERIALS AND METHODS

Microbial cultures. Two different mixed cultures, adapted to methane production from glucose were used. One culture (A) was originally obtained from a sewage sludge digester and had been cultivated continuously for 4 years at $35^{\circ}C$ on a glucose-based medium. This culture was adapted to $55^{\circ}C$. The other glucose-adapted culture (B) was obtained from a thermophilic ($55^{\circ}C$) pilot scale digester treating wastes from sugar-beets containing complex carbohydrates. As a third inoculum (C) mesophilic sewage sludge, without adaption to any laboratory substrates, was used.

Cultivation media. Glucose medium (g/litre): Glucose, 16; yeast extract, 4.0; NH_4Cl , 4.0; $Na_2HPO_4 \cdot 2H_2O$, 1.6; KH_2PO_4 , 0.15; $MgSO_4 \cdot 7H_2O$, 0.4. Trace

elements (mg/l): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.60; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.66; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.18; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.18; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.14; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.14. The substances were dissolved in distilled water and the medium was boiled and allowed to cool in a N_2 -atmosphere. In mixed acid media the glucose was replaced by 10 g/l each of acetic, propionic and butyric acids. In acetate medium the glucose was replaced by 22 g/l NaAc.

Fermentor cultivations. The adaption of the mesophilic glucose enrichment (A) to 55°C and the stepwise adaption of the thermophilic inoculum (B) at 55°C to extreme thermophilic temperatures was made in semi-continuous fermentors. Glucose-substrate was added continuously (0.5-1.5 g/l·d) and fermentor liquid removed intermittently. The fermentors (Chemoferm 4 litre, New Brunswick 2 litre) were sparged with N_2 or 30 % CO_2 /70 % N_2 ; all gases were passed through a $\text{CuO/Cu}_2\text{O}$ - column at 360°C to remove oxygen traces. The cultures were mixed by impellers at 600-800 rpm. pH was measured continuously with glass-calomel electrodes and controlled at 6.8 by automatic addition of 4M NaOH or 4M HCl. Redox potential was measured continuously with platinum-calomel electrodes.

The activity of methanogenic bacteria at different pCO_2 (0-1 bar) and temperatures (55 - 70°C) was measured as rates of acetic acid utilization and methane production in batch fermentors with rapid mixing, pH-control (6.8) and E_h -measurement. At each temperature tested, four fermentors sparged with N_2 , 30 % CO_2 /70 % N_2 , 60 % CO_2 /40 % N_2 and CO_2 , respectively, were inoculated with 1000 ml inoculum B from a continuous glucose-fermentor adapted to the actual temperature and 200 ml acetate substrate. Rates of acetic acid degradation and methane production were measured. Degradation of acetic, propionic and butyric acid by methanogens and acetogens were studied by using mixed acid substrate in batch fermentors at 55°C .

Analytical procedures. Fatty acid analysis was carried out by a gas chromatographic method described previously (Hansson and Molin 1981 a). Gas analyses (N_2 , CH_4 , CO_2) were carried out by gas chromatography

according to van Huyssteen (1967). All gases from the fermentors were cooled to room temperature before sampling. Glucose was measured by the hexokinase method (Schmidt 1961)

RESULTS AND DISCUSSION

Development of thermophilic cultures. The thermophilic inoculum (B) from the sugar-beet waste digester was transferred to a 55°C fermentor with N₂-sparging and continuous addition of glucose-substrate (around 1.5 g/l·d). As expected, methane production started immediately. The glucose was completely utilized and no acids accumulated.

This experiment was repeated, but after the inoculum had been stored at room temperature for 5 months. The fermentor was sparged with 30 % CO₂/70 % N₂ and the temperature was immediately increased to 55°C. Methane production from glucose started at once but with a temporary accumulation of acetic acid (500 mg/l). After 3 days with a continuous addition of glucose-substrate (loading-rate 1.0 g glucose/l·d) the substrate was completely utilized.

Adaption of the mesophilic culture (A) was also done in a fermentor sparged with 30 % CO₂/70 % N₂ and an immediate increase in temperature to 55°C. This culture was originally from mesophilic sewage sludge, but cultivated on glucose-substrate for 4 years. However, methane production from glucose also started immediately in this case although with a temporary accumulation of acetic acid to 2000 mg/l. After 5 days of continuous culture on glucose-substrate at 1.0 g/l·d the substrate was completely utilized.

Moreover, direct adaption of mesophilic sewage sludge (C) to 55°C in a fermentor without gas sparging was tested. Just as in the other cultures, methane production started immediately. This culture was unstable during a two-week period with varying gas yields and some accumulation of acids. After this period gas yields were stable (70-80 % of substrate-carbon in gas) and acid levels were low.

These results suggest that mesophilic mixed cultures were quite easily

adapted to thermophilic conditions. Adaption was somewhat faster if the cultures were sparged with N_2 or 30 % CO_2 /70 % N_2 . Storing a thermophilic culture at room temperature had only small and temporary adverse effects.

One interesting question is why there are thermophilic bacteria in mesophilic cultures. There may be some thermophiles in mesophilic sewage sludge but after 4 years of continuous, mesophilic culture these should have been diluted. Anyway, even if there were some thermophiles left, these would require considerable time to grow to numbers sufficient for any substantial methane production. It therefore seems likely that the mesophilic bacteria in the cultures used in this work had the capacity to switch to thermophilic conditions with short lag periods.

Activity of acetogenic and methanogenic bacteria at 55°C. Decomposition of propionic and butyric acid by acetogenic bacteria and acetic acid by methanogens were studied in batch fermentors at 55°C sparged with 30 % CO_2 / 70 % N_2 . Three cultures were tested: The mesophilic culture at 35°C (A), this culture after adaption to 55°C and the thermophilic culture from a sugar-beet waste digester at 55°C (B). The results are shown in Fig. 1. Rates of acid degradation and yields of methane are compared in Table 1.

After only one week of adaption of the mesophilic culture (A) to 55°C this culture degraded acetic and butyric acid at about the same rate as the original mesophilic culture. Propionate-degrading organisms, however, did not seem to have the capacity to switch to thermophilic conditions during a three-week period. The thermophilic waste-culture degraded acetate and butyrate 2-3 times faster than the other cultures. However, since the cellmass of participating bacteria cannot be measured, it is not known if these differences are due to higher specific activity, e.g. because of the longer adaption period to thermophilic temperature, or due to more cells in the culture.

There was a significant difference in methane yields between mesophilic and thermophilic conditions. In mesophilic fermentations the methane yield

was 51 % of substrate-carbon in CH_4 . This is the theoretical maximum since fatty acids should be degraded to CH_4 and CO_2 in equal amounts. In the mesophilic culture adapted to 55°C the methane yield was only 24 %. Since propionate was not degraded this figure corresponds to 35 % of HAc + HBut degraded to CH_4 . The thermophilic waste-culture also had a low methane yield despite the rapid and complete degradation of acids.

Effects of pCO_2 and temperature ($55\text{--}70^\circ\text{C}$) on acetate degradation. The rate of acetate utilization at different pCO_2 (0, 0.3, 0.6, 1.0 bar) was measured at 55, 60, 65, and 70°C using the sugar-beet waste inoculum (B). The results are shown on Fig. 2.

Roughly the same effects of pCO_2 were found at 55°C and 60°C as has been reported from mesophilic experiments (Hansson and Molin 1981 b). Mesophilically, the mean rate of acetate degradation was 1300 mg/l·d in N_2 and 350 mg/l·d in CO_2 . Thermophilically, rates were somewhat higher but CO_2 caused the same degree of inhibition.

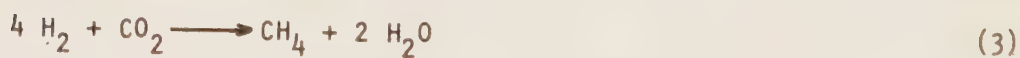
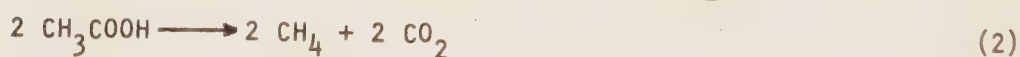
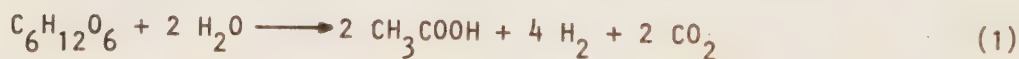
In 60 and 100 % CO_2 acetate degradation dropped rapidly at temperatures above 60°C and there was almost no methane production at 68 and 70°C . This is in agreement with the literature cited above. However, at low levels of CO_2 there was a considerable methane production from acetate also at 68 and 70°C .

These results suggest that CO_2 decreases the rate of acetate utilization by methanogens as well as temperature maximum for acetate degradation. Therefore, an interesting question is by which mechanism CO_2 affects methanogens. A mechanism for CO_2 -effects on bacterial spore germination was suggested by Enfors and Molin (1978). CO_2 was assumed to dissolve in cell membranes which would increase membrane fluidity and impair its function. Increase in temperature also has been found to increase membrane fluidity (McElhaney 1976). Therefore CO_2 would be expected to decrease growth rate and decrease temperature maximum for CO_2 -sensitive bacteria as was demonstrated by Enfors and Molin (1981) for growth of Bacillus cereus in 1 bar

CO₂. As for methanogens, the dependence of methanogenic activity on pCO₂ at 65-70°C and the general inhibiting effect of CO₂ on methane production from acetate support the theory on CO₂-effects on cell membranes.

Methane production at extreme temperatures (70-85°C). Since methane production from acetate was found to work at 70°C, provided a low pCO₂, further temperature increases were carried out in a N₂-sparged fermentor to establish the temperature maximum. These studies were made with continuous glucose-substrate addition since it had been a general observation during the fermentations at 55-70°C that continuous methane production from glucose during inoculum adaption was more rapid and stable than methane production from acetate in batch cultures at the same temperature. The effective volume of the fermentor increased continuously from 2200 ml to 4500 ml during the experiment.

A fermentation diagram for 50 days cultivation at 70-85°C is shown in Fig. 3. Since the levels of propionic and butyric acid were rather constant and no other intermediates were discovered by gas chromatography the degradation of glucose may be assumed to proceed according to the equations:



Substrate was added at 56 mg glucose/h which theoretically should yield 23 ml/h of both CH₄ and CO₂ (25°C), provided negligible amounts of acetate or other compounds were assimilated by bacteria and acetate was not completely oxidized to CO₂. While methane was produced from acids with around 100 % of the theoretical yield at 35°C and 50-70 % at 55°C (Table 1), yields at temperatures above 70°C were lower but still 20-30 % at 80°C. At 85°C the fermentation of glucose stopped, but traces of methane were still produced.

Apparently, the fermentation of glucose to acetic acid worked well up to 80°C. The glucose addition was 56 mg/h corresponding to 38 mg/h of acetate. Acetate was accumulated in the fermentor at an approximately constant rate, 21 mg/h, and around 3.5 mg/h were removed in the effluent. Therefore, the remaining acetate, 13.5 mg/h, should result in 5.6 ml/h of both CH₄ and CO₂, still provided degradation according to (1) - (3) and no assimilation of acetate. The considerably higher production of CO₂ compared to CH₄ at temperatures above 76°C may be explained by partly inhibited methane production from H₂/CO₂. A complete inhibition of this reaction but still methane production from acetate would result in 5.6 ml/h CH₄ and 21 ml/h CO₂. When these calculations are compared to the gas production data in Fig. 3, one conclusion is that methane was produced from acetate as well as H₂/CO₂ at 70-74°C and mainly from acetate at 76-80°C. According to the literature, temperature maximum for acetate degradation to methane is around 65°C. Therefore this work demonstrates a very strong effect of pCO₂ at high temperatures.

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Table 1 Rates of acid degradation and yields of methane of mesophilic and thermophilic methane production from fatty acids.

Culture	Temp	Acid degradation rate, mg/l·d			Y_{CH_4} %substrate-C in CH_4
		HAc	HPr	HBut	
Mesophilic	35	910 ^a	150	170	51
Mesophilic adapted to 55°C	55	850 ^a	0	160	24
Thermophilic waste	55	1620 ^a	170	750	30

^aCorrected for amounts of acetate formed from propionic and butyric acid during acetate degradation.

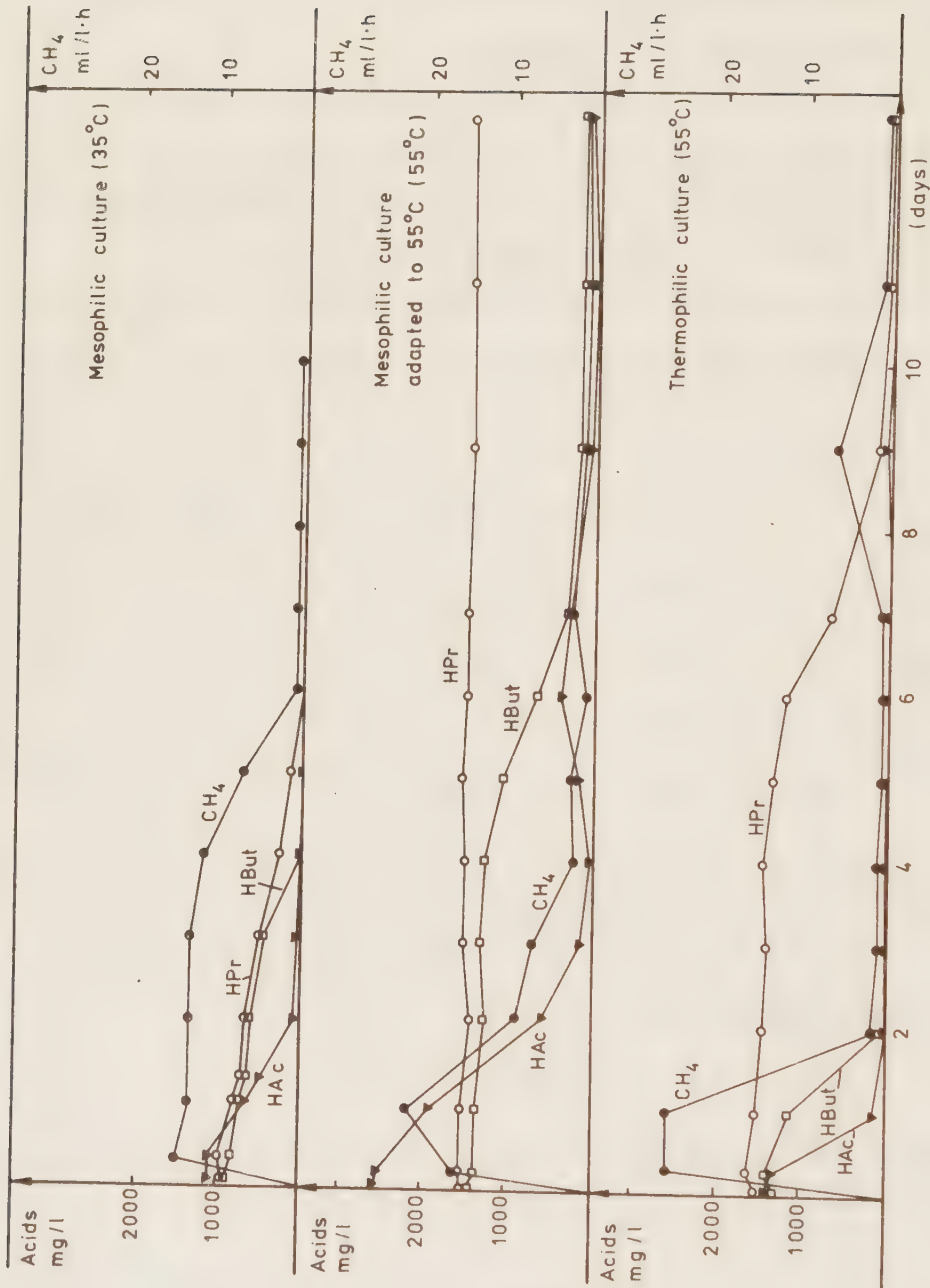


Fig. 1 Degradation of fatty acids to methane in mesophilic and thermophilic batch cultures.

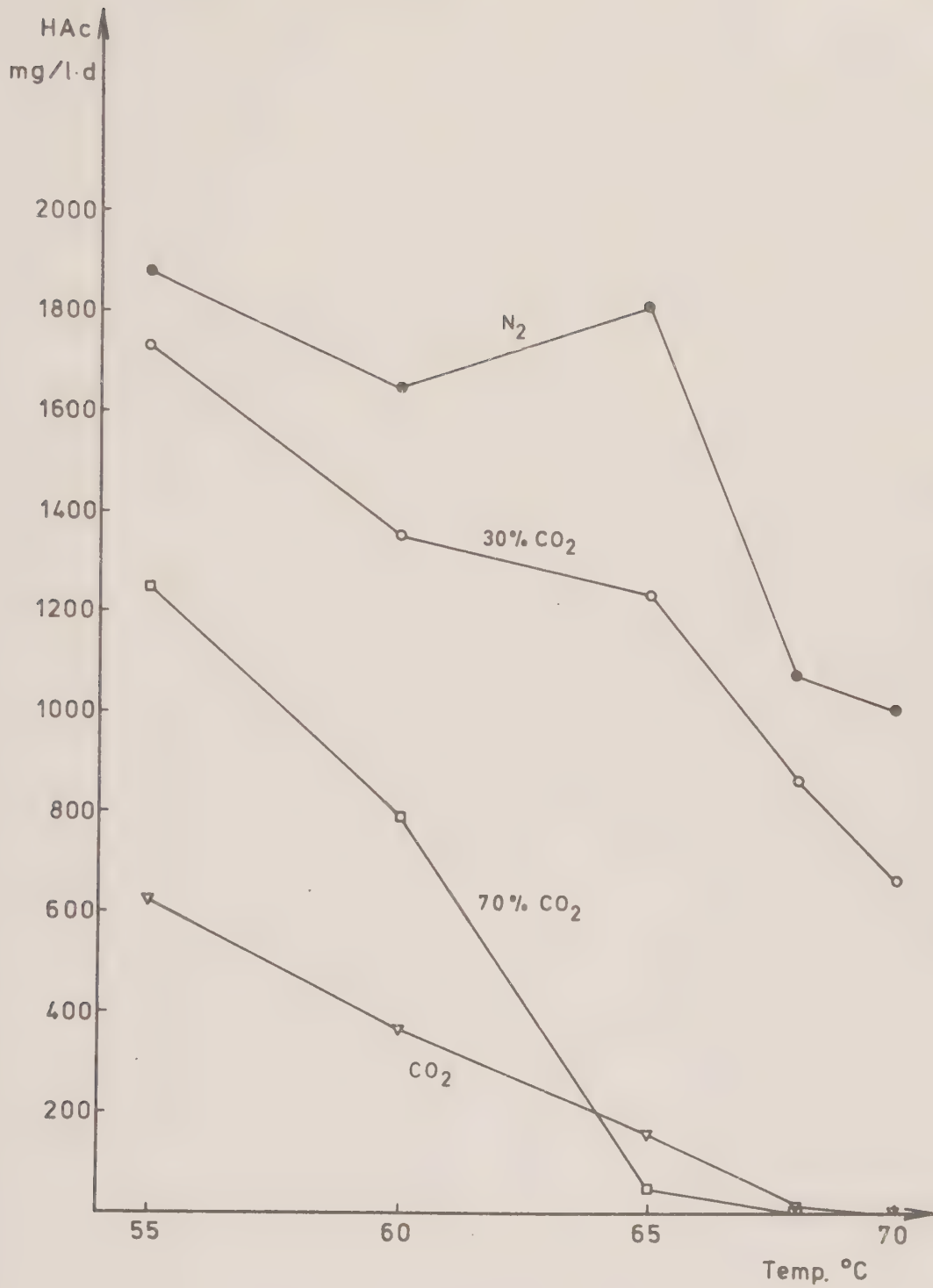


Fig. 2 Rates of acetate degradation as a function of $p\text{CO}_2$ (0-1 bar) and temperature (55-70°C).

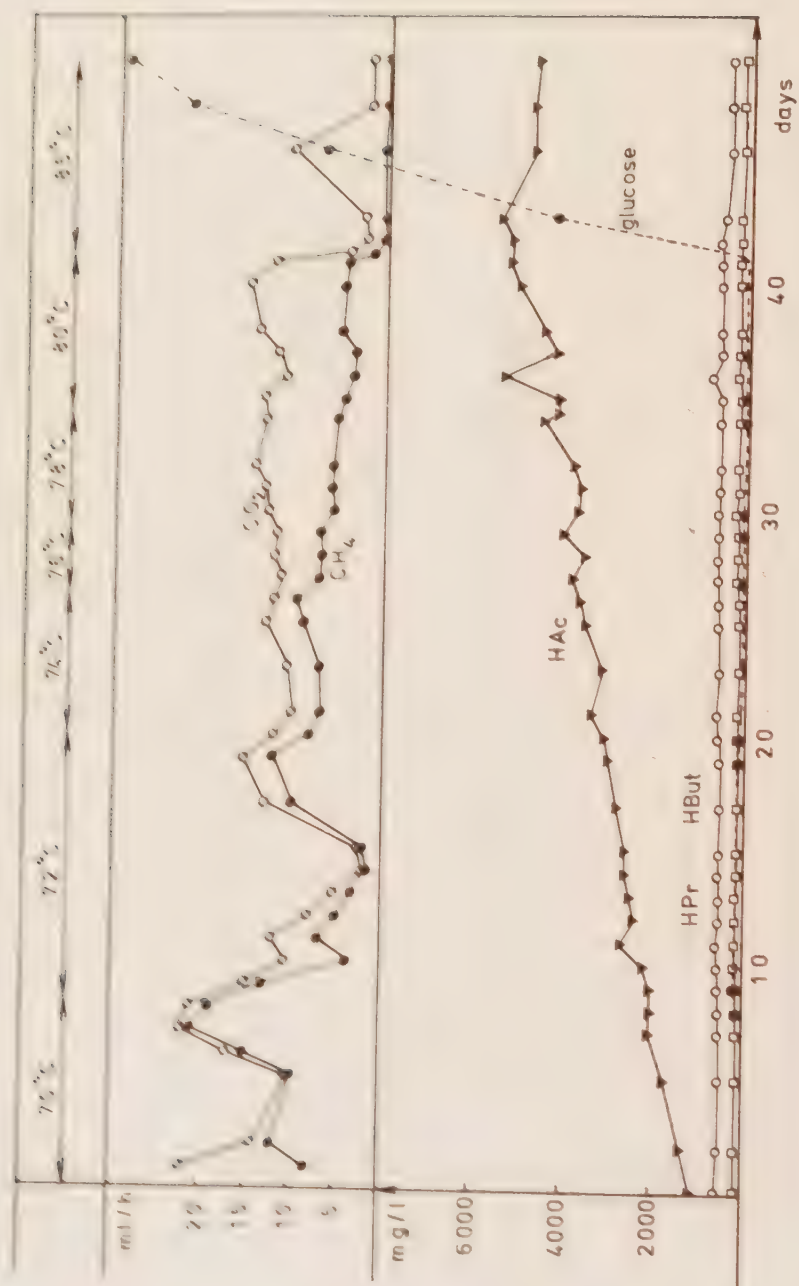


Fig. 3 Production of methane and carbon dioxide from glucose at extreme temperatures.

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METHANE PRODUCTION FROM MARINE, GREEN MACRO-ALGAE

Hansson G

ABSTRACT

Fermentation studies have been carried out to produce methane from green algae native to Scandinavian waters and suitable for large scale cultivation. Long term semi-continuous fermentations during mesophilic and thermophilic conditions were performed as well as batch fermentations in flasks and syringes. A mixed inoculum was prepared from sediments, rotting seaweed, sewage sludge and rumen contents. Methane production from the seaweed substrate, consisting of ground green algae without any nutrient additions, started immediately in this culture, mesophilically as well as thermophilically. Fermentations were carried out with retention times from 27 to 11 days and loading rates from 1.1 to 2.6 g VS_{added}/l·d. In the mesophilic fermentation gas yields were 250-350 ml CH₄/g VS_{added} and the VS-reduction was around 50-55 % at all tested retention times and loading rates. The level of volatile fatty acids was very low in this system. In the thermophilic digester gas yields were somewhat lower although the VS-reduction was around 50 % also in this system. The VFA-levels were higher and the culture more sensitive to disturbances. Thus no advantages were found with the thermophilic fermentation. In mesophilic batch fermentations the gas production was rather rapid and almost completed after 12-15 days, in agreement with the continuous fermentations. The gas yields in batch experiments were high, 350-480 ml CH₄/g VS_{added}.

INTRODUCTION

At present, in most applications of methane fermentations, organic wastes or wastewaters are stabilized with the simultaneous production of energy as methane. Future applications may include methane production from energy crops specially grown for the purpose of energy production. Crops suitable for methane production may be land-based as hay, lucerne and sugar-beets (1) or waterbased as algae, seaweeds and water hyacinths (2,3). Seaweeds as energy crops have certain advantages: use of large land areas - which may not be available - is avoided (4); marine crop yields are expected to

be considerably higher than on land although experience from large-scale cultivation is lacking (2,3); seaweeds do not contain lignins which are almost non-degradable during anaerobic conditions (5).

The most extensively studied system for utilizing marine biomass is the cultivation of U.S. West Coast kelp, Macrocystis pyrifera (6). Methane fermentation of Macrocystis pyrifera has been studied by Klass et al (7), while Troiano et al (8) have studied methane production from U.S. East Coast kelp, Laminaria saccharina. Work on methane production from biomasses including brown algae has been summarized by De Renzo (9) and Stafførd et al (10). Hanisak (11) reported on methane production from the red seaweed Gracilaria tikvahiae. In general, anaerobic digestion of these brown and red algae works but only around 50 % of the organic content (VS) in the algae is decomposed to gas resulting in gas yields of 200-300 ml CH₄/g VS_{added}. Pretreatment of algae, e.g. with heat and alkali, may increase the gas yields (8, 9, 12).

In this work methane production from the marine, green algae Ulva, Chaetomorpha and Cladophora has been studied. These algae are native to Scandinavian waters and recent small scale cultivation experiments suggest that yields of around 50 ton VS/ha·yr are possible (13). The purpose of this work was to demonstrate the feasibility of converting these green algae to methane and to determine basic fermentation parameters such as gas yields and retention times in conventional high-rate digestors.

MATERIALS AND METHODS

Inoculum preparation

To obtain a mixed culture with a great diversity of bacterial species samples were collected from a variety of anaerobic habitats. The final, mixed inoculum was composed of: 1) digesting sewage sludge (30 %), 2) rumen contents (20 %), 3) salt and brackish water sediments from four different sites (30 %), 4) rotting seaweeds from two sites (20 %).

Fermentor equipment

Two glass/stainless steel fermentors, total volume 2000 ml, effective volume 1200-1600 ml, were used. Temperatures were constant at 35° and 55°C, respectively, and the cultures were mixed by impellers at 300-500 rpm. pH was measured intermittently. The produced gas was collected in bags made from black rubber or aluminium film embedded in plastic films.

Substrate

Ulva, Cladophora and Chaetomorpha, harvested from the Baltic coast of southern Sweden, were ground to about 1 mm pieces. A small amount of water was added to facilitate grinding but no nutrients were added. The substrate was composed of a mixture of the three species in approximately equal amounts and was kept frozen. A volume sufficient for about one week of feeding the fermentors was thawed each time. Substrate was added to the fermentors manually every day or every other day depending on residence times. When the liquid volume in the fermentors had reached 1600 ml, 400 ml was removed and replaced by N₂.

Start-up procedure

The fermentors were sparged with N₂ and filled with 1000 ml inoculum and 50 ml substrate. Methane production started immediately and only low levels of acids accumulated temporarily. After 10 days, daily substrate addition was started with long retention times (50-60 days). After about 10 weeks the contents of the fermentors were sieved to remove large particles from the inoculum. This treatment did not upset the fermentation.

Batch fermentations

Batch fermentations were carried out in flasks and syringes. Two flasks were each inoculated with 500 ml of a mesophilic fermentor culture and 200 ml seaweed substrate. Two flasks were each inoculated with only the

500 ml fermentor culture, as a reference. The flasks were connected via butyl rubber tubing to gas-tight bags and kept at 35°C without shaking but mixed once daily. A method described by Viitasalo (14) was also used. Four 60 ml syringes were filled with 25 ml inoculum + 10 ml seaweed substrate or 25 ml inoculum only. Due to gas production the pressure in the syringe increased slightly causing the barrel to move and the produced gas volume could be read directly.

Analytical procedures

Total Solids (TS) and Volatile Solids (VS) were measured as described in Standard Methods (15).

Fatty acids were analyzed by gas chromatography using a Varian model 940 chromatograph with flame ionization detector, autosampler and CDS 101 integrator.

Column: 8 ft teflon column 1/16 inch by 1/8 inch with a 100 mm empty glass column at the injection end.

Support: Chromosorb 101.

Carrier gas: N₂ 30 ml/min, saturated with distilled formic acid.

Temperatures: Injector 190°C, column 175°C, detector 190°C.

Preparation

of samples: Fermentation liquids are acidified, centrifuged and then injected (0.5 µl) into the glass column. Most contaminants will adhere to the walls of the glass column which is regularly changed to a clean column.

Gas analyses (N₂, CH₄, CO₂) were carried out by gas chromatography according to van Huyssteen (16).

RESULTS AND DISCUSSION

Two fermentors were run continuously for about one year. One fermentation was mesophilic at 35°C, one was thermophilic at 55°C, after a start-up period at 60°C. Data from the fermentations are shown in Fig. 1 and Fig. 2.

The fermentors were treated identically except for the temperature. After the start-up procedure with very long retention times, as described above, the fermentors were run with gradually decreasing retention times and increasing loading rates. Analyses of VS/TS in the seaweed-substrate and in the fermentors, of the volatile fatty acids in the fermentors, and of the produced gas volumes and gas composition were made 1-2 times every week and gas production and VS-reduction were calculated. Mean values of data for 2 week-periods are shown in Fig. 1 and Fig. 2.

Retention times and loading rates

After start-up both fermentors were run at RT = 27 days for 25 weeks. Then followed 9 weeks at RT = 19 days, 6 weeks at RT = 16 days and 4 weeks at RT = 11 days. Loading rates depend on RT as well as substrate concentration. During the first 20 weeks of fermentation the seaweed-substrate had a mean TS-content of 10 % and a mean VS-content of 5.3 %. During the rest of the fermentations the dry matter content of the mixed seaweed substrate was lower with mean values of 5.2 % TS and 3.0 % VS. Therefore the loading rate, expressed as $\text{g VS}_{\text{added}}/\text{l}\cdot\text{d}$, was around 2 during the first 10 weeks at RT = 27 days and then decreased to 1.1 $\text{g}/\text{l}\cdot\text{d}$ as substrate concentrations decreased. Then stepwise increases in loading rates followed to final values of 2.6 $\text{g VS}/\text{l}\cdot\text{d}$ as a result of the lowered RT.

The mesophilic fermentor worked well at the tested loading rates and retention times. Possibly a slight decrease in gas yield occurred at RT = 11 days but no build up of acids or reduction of VS-utilization was noted. Therefore, retention times of 12-15 days should be suitable in methane production from green algae in mesophilic high-rate digestors. It should be possible, however, to increase loading rates further by using substrates with a higher VS-content. The observed possibility to use comparatively short retention times without gas yield reduction com-

pares well with literature data. To obtain high gas yields from seaweed and other biomasses retention times of 15-30 days are usually employed during mesophilic conditions (7, 9). The fraction of the organic material degraded to gas in the green algae used in this work was therefore relatively easily degradable.

During thermophilic conditions gas production from green algae worked at all tested retention times and loading rates. However, gas yields were lower and the performance of the fermentation poorer, as discussed below.

Gas yields and VS-reduction

When gas is produced from biomass on a 1-litre scale only small volumes of gas are formed daily. It is therefore essential to keep fermentors, tubings and gas collectors gas-tight to prevent leakage. In our work leakage from gas bags was especially crucial. Initially, commercially available black rubber balloons were used, but these were not sufficiently gas-tight. Gas bags were then made by welding sheets of plastic/aluminium films. When these bags replaced the rubber balloons (after 18 weeks), the observed gas production increased immediately (Figs 1 and 2). However, these bags were fragile and easily punctured and gas production decreased again. Therefore a new and heavier type of bags was constructed and better methods to empty the bags employed (after 40 weeks) resulting in an increase in the observed gas production again.

Gas phase composition was usually around 60 % CH_4 and 40 % CO_2 . Theoretically, equal amounts of CH_4 and CO_2 should be produced (4) but some CO_2 will dissolve in the fermentation liquid. Methane yields - with gas-tight bags - during mesophilic conditions were 250-350 ml $\text{CH}_4/\text{g VS}_{\text{added}}$ at all retention times between 11 and 27 days. Total gas yields were then 400-600 ml gas/g VS_{added} . The VS-reduction was around 50-55 % irrespective of retention times and loading rates and therefore methane yields may be

expressed as 450-650 ml $\text{CH}_4/\text{g VS}_{\text{degraded}}$. The volumetric methane production was 200-250 ml $\text{CH}_4/\text{l}\cdot\text{d}$ at 16 days RT and 300-370 ml $\text{CH}_4/\text{l}\cdot\text{d}$ at 11 days RT.

The obtained methane yields from green algae were somewhat higher than the yields of 200-300 ml $\text{CH}_4/\text{g VS}_{\text{added}}$ obtained from brown and red algae according to the literature reviewed above. However, VS-reduction was about the same. For comparison, in methane production from various crops and organic wastes gas yields are usually 200-500 ml gas/g organics added at 60-70 % methane (1, 17, 18).

In the thermophilic fermentor the VS-reduction was generally around 50 %, as in the mesophilic fermentor, and roughly the same gas yields would be expected. As seen in Fig. 2 there are several periods of low gas yields but these may be explained by gas leakages or temporarily low temperatures due to power failure and other technical problems. However, even during periods when the thermophilic digester worked well gas yields were somewhat lower than in the mesophilic digester, especially at the shorter retention times.

VFA, pH, E_h

In the mesophilic digester the concentrations of volatile fatty acids (VFA) were generally very low, below 100 mg/l. At start-up the VFA-level was temporarily 500-1000 mg/l, but after this period no build-up of VFA was noted due to sudden increase in loading rates, technical problems with mixing and temperature control etc. In the thermophilic digester, on the other hand, VFA-levels were generally higher, often around 1000 mg/l. This is in agreement with several reports on thermophilic digestion, as reviewed by Buhr and Andrews (19). This culture was also much more sensitive to disturbances resulting in periods with very high VFA-levels.

pH was measured every week but not controlled except for one addition of acid (after 41 weeks). Generally pH was in the range 7.0-7.7. Mean values for the mesophilic fermentor were around 7.2 and for the thermo-

philic fermentor around 7.4. In both fermentors there was a tendency towards higher pH at shorter retention times.

The redox potential (E_h) was measured occasionally. E_h -values of -300 to -320 mV were found in both fermentors corresponding to electrode potentials against the standard calomel electrode of -544 to -564 mV.

Mesophilic - thermophilic digestion

Both fermentors produced methane immediately after the addition of algal substrate. It is notable that all bacteria in inoculum and substrate were from mesophilic or temperate environments but all the same, thermophilic methane production at 60°C started at once. However, no advantages of thermophilic digestion were found in this work over the mesophilic system. Gas yields were somewhat lower, VFA-levels higher and the thermophilic digester was also more sensitive to disturbances. Considerable advantages of the thermophilic digester would be required to warrant the heating costs to maintain the high temperature and no such advantages were found. However, it should not be ruled out that changes in fermentation conditions and selection of thermophilic inocula might improve a thermophilic system.

Gas yields in batch fermentations

Batch fermentations were carried out in four flasks and four syringes as described above. Accumulated methane production, i.e. methane production from flasks/syringes containing inoculum + seaweed substrate less methane production from inoculum alone, is shown in Fig. 3. Data is shown only from one bottle due to air leakage into the other bottle.

Methane production from algae in batch fermentations was rather rapid and the gas production was almost completed after 12-15 days. This is in agreement with the semicontinuous fermentations where gas yields were about the same for all retention times down to 11 days. However, the methane yields in batch cultures were higher, 350-480 ml $\text{CH}_4/\text{g VS}_{\text{added}}$,

compared to 250-350 ml $\text{CH}_4/\text{g VS}_{\text{added}}$ in semi-continuous fermentations. This difference in gas yields should be due to a greater VS-reduction in batch fermentations although it was not possible to measure the exact degree of VS-reduction. The reasons for a higher VS-reduction and gas yield in batch fermentations are not known. One possible explanation may be negative effects of the comparatively vigorous mixing in the semi-continuous fermentations. However, mixing is usually considered essential (19) and no adverse effects of vigorous mixing were found in methane production from glucose or fatty acids (20). Possibly, vigorous mixing may decrease gas yields from complex and particular substrates.

CONCLUSIONS

- Long term, continuous methane production from a mixture of ground, green algae was possible. No additions to the seaweed-substrate were necessary.
- A rapid start-up was achieved by using a mixed inoculum from a variety of habitats known for methane production and by regular feeding with seaweed-substrate at low loading rates.
- The degradation of green algae was rather rapid and retention times of 12-15 days should be suitable.
- Methane yields from green algae were somewhat higher than yields from brown and red algae reported in the literature. In continuous methane fermentation yields were 250-350 ml $\text{CH}_4/\text{g VS}_{\text{added}}$.
- VS-reduction was around 50-55 %, in agreement with most literature data.

- No advantages were found with thermophilic methane production from algae, over mesophilic fermentation.
- This work was carried out with conventional high-rate digestors. By using more advanced fermentation techniques and possibly pre-treatment of the substrate further improvement of methane yields and fermentation kinetics should be possible.

ACKNOWLEDGEMENTS

Thanks are due to Torgny von Wachenfeldt, Dept. of Marine Botany, Univ. of Lund for financial support and for providing algal substrates. Skilful technical assistance by Katarina Åkesson, Ann Barthel, Karl Mohlin and Jan Sjöholm is greatly acknowledged.

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Fig. 1 Mesophilic methane production from green algae in semi-continuous culture.

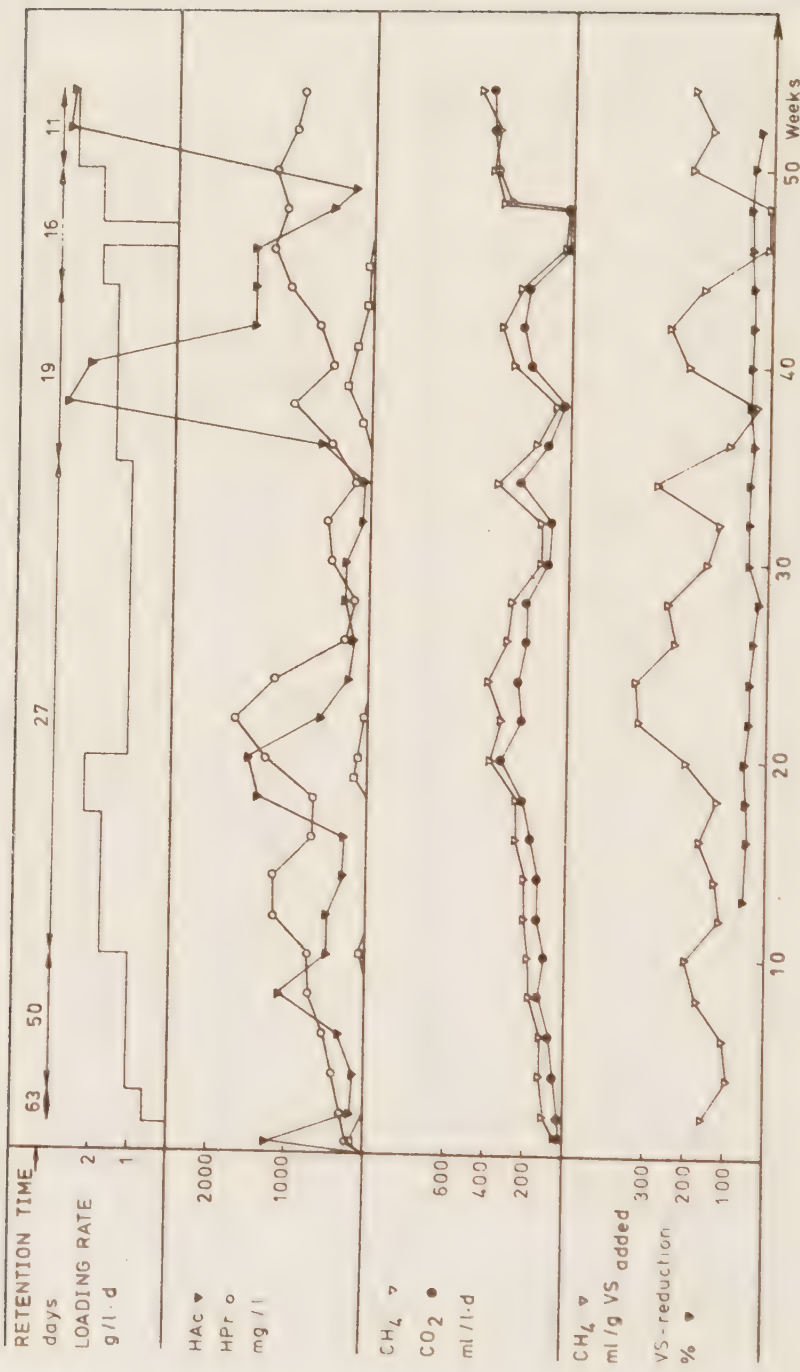


Fig. 2 Thermophilic methane production from green algae in semi-continuous culture.

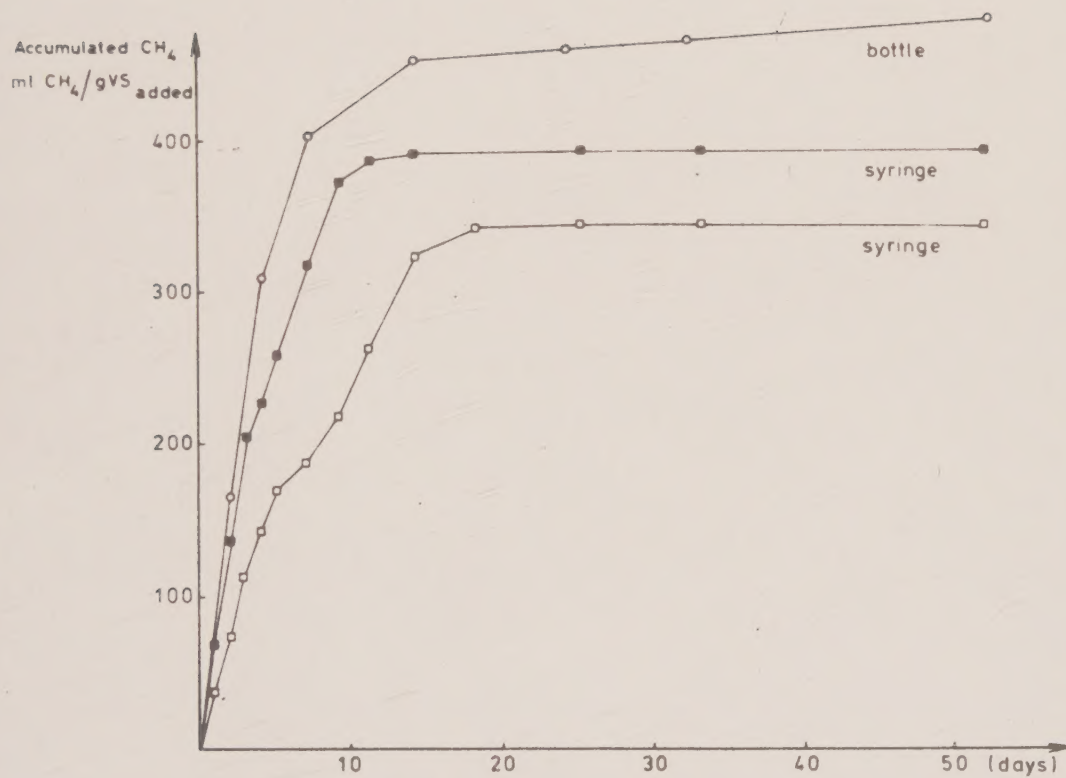


Fig. 3 Accumulated methane production in batch fermentations of green algae.

